



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US93/03650 <b>(22) International Filing Date:</b> 16 April 1993 (16.04.93)  <b>(30) Priority data:</b> 869,697                      16 April 1992 (16.04.92)                      US  <b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 101 N. Wilmot Road, Suite 600, Tucson, AZ 85711-3335 (US).  <b>(72) Inventors:</b> PIDGEON, Charles ; 515 Evergreen Lane, West LaFayette, IN 47906 (US). MARKOVICH, Robert, J. ; 27 Rachel Court, Franklin Park, NJ 08823 (US).		<b>(74) Agent:</b> SCOTT, Anthony, C.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).  <b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NOVEL ACYLATED PHOSPHOLIPID DRUGS  <div style="text-align: center; margin: 20px 0;"> <math display="block">  \begin{array}{c}  \text{O} \\  \parallel \\  \text{O} - \text{P} - \text{OR} \\    \\  \text{OH} \\  \text{CH}_2 - \text{CH} - \text{CH}_2 \\    \quad   \\  \text{OR}_1 \quad \text{OR}_2  \end{array}  </math> </div> <b>(57) Abstract</b> <p>The invention relates to a compound having formula (A) or pharmaceutically acceptable salts thereof, wherein one of R<sub>1</sub> and R<sub>2</sub> is a heteroatom fatty acid acyl group having 13-14 carbon atoms in the principal chain and up to a total of 18 carbon atoms, while the other is hydrogen, a heteroatom of fatty acid acyl group containing 13-14 carbon atoms in the principal chain and up to a total of 18 carbon atoms or an acyl group of a fatty acid containing 4-26 carbon atoms in the principal chain and up to a total of 30 carbon atoms and R is a naturally occurring polar group characteristic of a glycerolphospholipid isolated from endogenous sources. This invention also relates to the use of the compounds of the present invention for inhibiting retrovirus infections and for the treatment of AIDS or AIDS related diseases.</p>		

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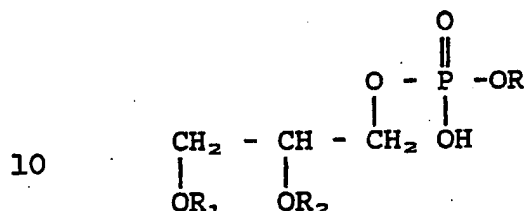
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NOVEL ACYLATED PHOSPHOLIPID DRUGS

The present invention relates to novel glycerol phospholipids useful as a drug for the treatment of AIDS and AIDS related complex. More specifically, the present invention is directed to a compound of the formula:



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or pharmaceutically acceptable salts wherein

one of  $R_1$  and  $R_2$  is a heteroatom fatty acid acyl group containing 13-14 carbon atoms in the principal chain and up to a total of 18 carbon atoms, while the other is hydrogen, a heteroatom fatty acid acyl group containing 13-14 carbon atoms in the principal chain and up to a total of 18 carbon atoms or an acyl group of a fatty acid containing 4-26 carbon atoms in the principal chain and up to a total of 30 carbon atoms and

$R$  is a naturally occurring polar group characteristic of a glycerophospholipid isolated from endogenous sources.

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The present compounds are useful in the treatment of AIDS. More specifically, the compounds of the present invention possess anti-viral activity especially anti-retroviral activity. Thus, these compounds of the present invention are useful in combatting and/or retarding the growth of retroviruses, such as the human immunodeficiency virus (HIV). As such, the compounds of the present invention are useful

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lin treatment of acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC).

The effectiveness of the compounds of the present invention are illustrated in the following 5 figures, which are discussed in more detail hereinbelow.

Figure 1 depicts the anti-HIV activity in T-cells of AC1, AC2, 1-(12-methoxydodecanoyl)-sn-3-glycerophosphatidylcholine and 12-methoxydodecanoic acid. Results are plotted on mM scale for direct comparison. Toxicity of the compounds in the CEM cells is labelled above each bar as toxic or non-toxic. The % reduction in the direct cytopathic effect of the virus (CPE) is represented on the Y-axis.

Figure 2 shows the anti-HIV activity in macrophages of L-AC1, L-AC2 and 12MO measured with an HIV p24 antigen assay.

Figure 3 depicts the anti-HIV activity of L-AC2 and 12MO in MT-4 cells using syncytial cell assays.

Figure 4 depicts the antiviral activity of L-AC2 and 12MO in peripheral blood monocytes (PBMC's) measured by reverse transcriptase assay. The results are depicted as the % of control.

Figure 5 demonstrates the toxicity activity of L-AC2 and 12MO used to evaluate the concentration of drug that kills 50% of MT-4 cells in the absence of virus. Dose response curves were used to evaluate the concentration of drug that kills 50% of MT4 cells. This 50% effective toxicity to cells is denoted as TC50 in each graph.

Figure 6 depicts the potent anti-HIV synergism when L-AC2 and AZT are concurrently administered.

1           Figure 7 depicts the anti-HIV activity of D-AC2 (unnatural glycerophosphatidyl choline configuration) and phosphatidylcholine (PE) analogs, L-PE-1 and L-PE-2 compared with 12MO.

5           Figure 8 depicts the stability of various anti-HIV phospholipids, L-PE1, L-AC2, D-AC2 and L-PE2 in fresh blood at 38°C.

          As used herein, the pharmaceutically acceptable salts include the acid and basic salts.  
10 Basic salts for pharmaceutical use are potassium, sodium, calcium, magnesium, zinc and the like. Suitable acids include for example, hydrochloric, sulfuric, nitric, benzenesulfonic, toluenesulfonic, acetic, maleic, tartaric and the like which are pharmaceutically  
15 acceptable.

          The term "lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms and may be straight chain or branched. It includes such groups as methyl, ethyl, propyl, isopropyl, butyl, sec-butyl,  
20 isobutyl, t-butyl, pentyl, amyl, hexyl and the like. The preferred lower alkyl group is methyl.

          The term "fatty acid" shall mean a carboxylic acid derived from or contained in animal or vegetable fat or oil. Said fatty acids may be saturated or  
25 unsaturated and are composed of a chain of alkyl groups containing from 4 to 26 carbon atoms, usually even numbered. The fatty acids are characterized by a terminal carboxy group. They also may contain a hydroxy group or a second carboxy group. It is preferred that  
30 the second carboxy group, when present, is located at the omega (last) carbon position of the principal chain.

1           Examples of fatty acids are described in  
SCIENTIFIC TABLES, 7th Edition, published by CIBA-Geigy  
Limited, Basle Switzerland, p. 365-372 (1970), and the  
contents are incorporated by reference as if fully set  
5 forth herein. These examples include the natural  
product fatty acids, such as propionic acid, n-butyric  
acid, valeric acid, caproic acid, enanthic acid,  
caprylic acid, pelargonic acid, capric acid, undecylic  
acid, lauric acid, margaric acid, stearic acid,  
10 nondecylic acid, arachidic acid, heneicosanoic acid,  
behenic acid, tricosanoic acid, lignoceric acid,  
pentacosanoic acid, cerotic acid, acrylic acid, trans-  
( $\alpha$ )-crotonic acid, iso( $\beta$ )-crotonic acid,  $\Delta^2$  hexenoic  
acid,  $\Delta^4$ -decenoic acid,  $\Delta^9$ -dodecanoic acid,  $\Delta^4$   
15 dodecanoic acid,  $\Delta^6$ -dodecanoic acid, tsuzuic acid,  
physteric acid, myristoleic acid, palmitoleic acid,  
petroselinic acid, oleic acid, eladic acid, trans- and  
cis-vaccenic acid,  $\Delta^{12}$ -octadecenoic acid, gadoleic  
acid.  $\Delta^{11}$ -eicosenoic acid, cetoleic acid, erucic acid,  
20 brassidic acid, selacholeic acid, ximenic acid, sorbic  
acid, linoleic acid, hiragonic acid,  $\alpha$ -eleosteric acid,  
 $\beta$ -eleostearic acid, linolenic acid, stearidonic acid,  
arachidonic acid, behenolic acid, isobutyric acid,  
isovaleric acid, tiglic acid, isomyristic acid,  
25 anteiomargic acid, tuberculostearic acid, phytanic acid,  
myocolipenic acid, myococeranic acid, and the like.  
Preferably, the term fatty acid as used herein shall  
contain 10 to 22 carbon atoms, and more preferably shall  
contain 13 to 18 carbon atoms. Most preferably, the  
30 fatty acid shall contain 4-8 carbon atoms or 13-15  
carbon atoms.

1 As used herein, the term "fatty acyl of a fatty acid" is defined as a fatty acid in which the carboxy terminus is replaced by an acyl group (  $-\overset{\overset{\text{O}}{\parallel}}{\text{C}}-$  ).

5 In other words, said term has the formula  $\overset{\overset{\text{O}}{\parallel}}{\text{C}} - \text{R}_7$ ,

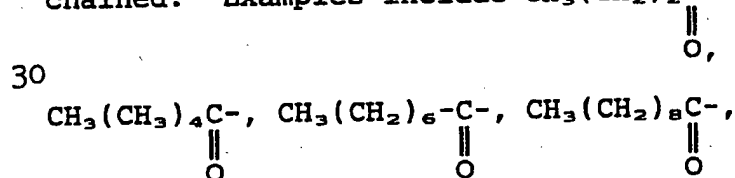
wherein  $\text{R}_7$  is a hydrocarbyl group as defined herein. For example, the fatty acyl of myristic acid is



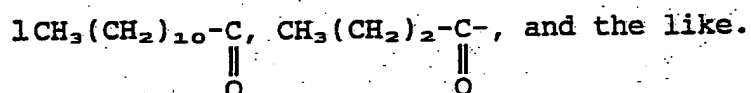
It is preferred that the fatty acids acyl group contains 4-26 carbon atoms. Besides the acyl group, (C), the fatty acid acyl group may contain

15  $\overset{\overset{\text{O}}{\parallel}}{\text{C}}$  unsaturation, e.g., double or triple bonds between the carbon atoms, but it is preferred that the fatty acid acyl group contains single bonds between the carbon atoms. In fact, except for the acyl, it is preferred  
20 that this group is a hydrocarbyl group, as defined herein. It is especially preferred that the hydrocarbyl group be saturated and contain 4-16 carbon atoms, and most preferably 4-14 carbon atoms. Finally, it is  
25 preferred that the group contains an even number of carbon atoms.

The fatty acyl group may be straight chained or branched, but it is preferred that it is straight chained. Examples include  $\text{CH}_3(\text{CH}_2)_2\overset{\overset{\text{O}}{\parallel}}{\text{C}}-$



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The term "heteroatom fatty acid" is a biologically active fatty acid analog of myristic acid chosen from a saturated or partially unsaturated fatty acid containing 13-14 carbon atoms, wherein at least one methylene group normally present at position 4 to 13 is replaced by at least one oxygen or sulfur atom. It is preferred that the heteroatom fatty acid contain 1-3 oxygen or sulfur atoms or a combination thereof. It is especially preferred that only one methylene group is replaced by oxygen or sulfur and it is most preferred that said methylene group is replaced by oxygen.

Preferred heteroatom fatty acids employable in the present invention include but are not limited to: 11-(ethylthio)undecanoic acid [ $\text{CH}_3\text{CH}_2\text{S}(\text{CH}_2)_{10}\text{COOH}$ ]; 5-(octylthio)pentanoic acid [ $\text{CH}_3(\text{CH}_2)_7\text{S}(\text{CH}_2)_4\text{COOH}$ ]; 11-(methoxy)undecanoic acid [ $\text{CH}_3\text{O}(\text{CH}_2)_{10}\text{COOH}$ ]; 11-(ethoxy)undecanoic acid [ $\text{CH}_3\text{CH}_2\text{O}(\text{CH}_2)_{10}\text{COOH}$ ]; 12-(methoxy)dodecanoic acid [ $\text{CH}_3\text{O}(\text{CH}_2)_{11}\text{COOH}$ ]; 10-(propylthio)decanoic acid [ $\text{CH}_3(\text{CH}_2)_9\text{S}(\text{CH}_2)_9\text{COOH}$ ]; 10-(propoxy)decanoic acid [ $\text{CH}_3(\text{CH}_2)_2\text{O}(\text{CH}_2)_9\text{COOH}$ ]; 11-(1-butoxy)undecanoic acid [ $\text{CH}_3(\text{CH}_2)_3\text{O}(\text{CH}_2)_{10}\text{COOH}$ ]; [10-(2-propynyloxy) decanoic acid [ $\text{HC}\equiv\text{CCH}_2\text{O}(\text{CH}_2)_9\text{COOH}$ ]; and the like.

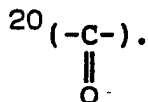
Additionally, the term heteroatom fatty acid is a saturated or unsaturated  $\text{C}_{13}$  to  $\text{C}_{14}$  fatty acid which is substituted by halo, hydroxy, alkoxy, mercapto or alkylthio. More preferably, the heteroatom fatty acid is a saturated or unsaturated fatty acid containing 13 to 14 carbon atoms which is substituted with halo or



1hydroxy. More preferred heteroatom fatty acids are saturated or unsaturated fatty acids which contain 13 to 14 carbon atoms and are substituted by chloro, bromo or hydroxy. Still more preferred are saturated or unsaturated fatty acids which contain 13 to 14 carbon atoms which are substituted by chloro, bromo or hydroxy at the 2-position.

Additionally, it is to be understood, within the spirit and scope of the present invention, that the term heteroatom fatty acid may also be a  $C_{13}$  to  $C_{14}$  saturated or unsaturated fatty acid wherein a methylene group normally at carbon position 5 to 12 is replaced by oxygen or sulfur, and further, said fatty acid may be substituted, preferably at the 2-position, by halo, hydroxy, alkoxy, mercapto or alkylthio.

The term "alkyl heteroatom fatty acid acyl group" as defined herein is defined as a heteroatom fatty acid containing no multiple carbon-carbon bonds in which the carboxy terminus is replaced by an acyl group



As used herein, the term halo shall mean one or more members of Group VII A of the periodic table, including fluorine, chlorine, bromine, and iodine; most preferably, fluoro or chloro and especially bromo.

The term alkoxy denotes an o-alkyl group, wherein alkyl is defined hereinabove. Examples of alkoxy are methoxy, ethoxy, propoxy, butoxy, pentoxy, hexoxy, and the like.

The term alkylthio is defined as an alkyl, as hereinbefore defined, containing a thio group.

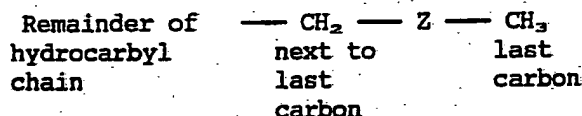
1 The term mercapto shall mean HS.

The term "omega (w) carbon" refers to the last carbon in the principal chain.

The term penultimate carbon refers to the next 5to last carbon on the principal chain. For example, in the decyl substituent, C<sub>10</sub> is the omega carbon while C<sub>9</sub> is the penultimate carbon.

The term "heteroatom is bonded to the penultimate carbon" or any equivalence thereof, means 10that the heteroatom is bonded between the omega and penultimate carbon. For example, if Z is a heteroatom, and if it is stated that Z is bonded to the penultimate carbon, this means that in the principal chain, Z is located between the last and the next to last carbon:

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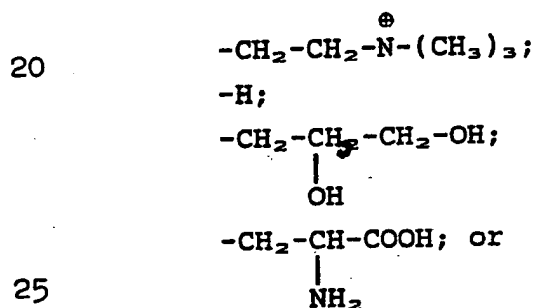


As defined herein, R is part of the polar head 20group and is a distinguishing portion of a glycerophospholipid. The polar group may be naturally occurring or analogs thereof. There are many types of polar groups on glycerol phospholipids found in nature. The more common R groups thereon are inositol, 25ethanolamine, choline and serine. But recently other head groups have been found, e.g., N-methyl ethanolamine, N,N-dimethyl ethanolamine, (See Casal et al., Biochemica et Biophysica Acta, 1983, 735, 387-396), and sulfocholine, (See Mantsch et al. Biochemical et 30Biophysica Acta, 1982, 689, (63-72) and the like. The present invention contemplates these groups found in

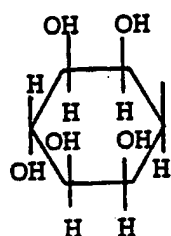
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1naturally occurring glycerophospholipids and analogs thereof.

The polar groups used herein have a dipole moment. These polar groups may contain heteroatoms, 5such as O, S or N or P. In fact, they may contain more than 1 heteroatom, e.g., 2, 3, 4, 5 or 6 - 9. Thus, there may be as many as 8 or 9 heteroatoms present in the R group. The polar head group may be a natural sugar (e.g., inositol) or combination of natural sugars 10(e.g., inositol-glycon) or the R group may consist of an alkylene chain in which a methylene group is replaced by a heteroatom or a heteroatom lower alkyl (N-CH<sub>3</sub>, e.g.) or heteroatom diloweralkyl [e.g. S(CH<sub>3</sub>)<sub>2</sub>], or if the heteroatom is nitrogen, a triloweralkyl heteroatom. 15Examples include inositol, ethanolamine, choline, sulfocholine, serine, a N-methyl ethanolamine, N,N-dimethyl-ethanolamine, and the like. The preferred R groups are



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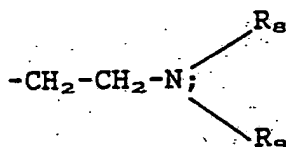
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, or

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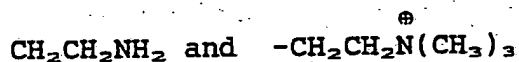
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wherein  $\text{R}_8$  and  $\text{R}_9$  are independently hydrogen or lower alkyl, and preferably hydrogen. The most preferred are



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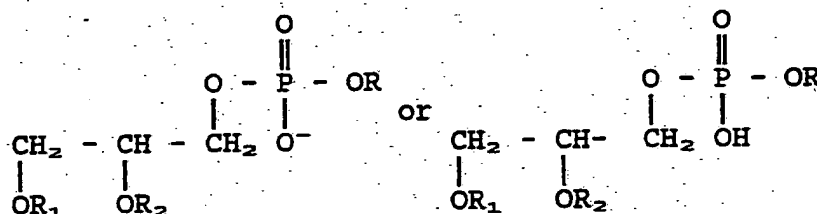
The prefix "sn-" as employed herein is used to denote the carbons of the glycerol backbone of the fatty acid according to the stereospecific numbering system established for lipid nomenclature. In other words, sn-1 denotes the carbon at the first position, sn-2 denotes

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the carbon at the second position, etc.

The phospholipid ester depicted hereinabove can exist in two forms,

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The term "basic salts" contemplates the former form, wherein basic salts are as defined herein. Unless specified to the contrary, the drawing of one form also contemplates to the other form.

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The carbon atom at the sn-2 position of the phospholipid ester depicted hereinabove contains an asymmetric center. Thus, the phospholipid ester as well as carbon atoms may exist in two stereochemical configurations, the L-stereoisomeric form (the natural

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1 configuration) or the D- form. Both stereoisomeric  
forms are contemplated by the present invention.

It is to be noted that when  $R_2$  or  $R_1$  is  
hydrogen, these compounds represent the  
5 glyosphospholipids of the present invention.

Of course, there may be additional chiral  
centers present on the  $R$ ,  $R_1$  and  $R_2$  groups, which also  
gives rise to various stereoisomeric forms. These  
various stereoisomeric forms are also contemplated to be  
10 within the scope of the invention. Therefore, all of  
the various configurations around each chiral center  
present in the phospholipid compounds of the present  
invention, including the various enantiomers and  
diastereomers as well as racemic mixtures and mixtures  
15 of enantiomers and/or diastereomers of the compounds of  
the present invention, either singly or in combination,  
are contemplated by the present invention.

It is preferred that when  $R_2$  or  $R_1$  is other  
than hydrogen,  $R_2$  and  $R_3$  are not branched, but are  
20 straight chained.

It is preferred that the hydrocarbyl group of  
the heteroatom fatty acid, i.e., the aliphatic portion  
of the heteroatom fatty acid, is saturated. When  $R_2$  or  
 $R_1$  is other than hydrogen, they may be heteroatom fatty  
25 acid acyl group or an acyl group of a fatty acid, as  
defined herein.

When  $R_1$  and  $R_2$  are a fatty acid acyl group,  
the fatty acid acyl group may have, in one embodiment,  
4-8 carbon atoms, or in another embodiment 13-14 carbon  
30 atoms. It is also preferred that the alkyl fatty acid  
acyl group be a hydrocarbyl fatty acid acyl group.  
Additionally, in an embodiment of the present invention,

1 the fatty acid acyl group has a carboxy substituent at  
the omega carbon. It is preferred also that  $R_1$  and  $R_2$   
be straight chained.

5 When  $R_2$  or  $R_1$  is a heteroatom fatty acid acyl  
group, then this group has the characteristics as  
defined herein. It is preferred that the heteroatom  
fatty acid acyl group contain no carbon-carbon multiple  
bonds. In one embodiment, it is preferred that  $R_2$  and  
10  $R_1$  consist of a hydrocarbyl moiety attached to the acyl.  
It is preferred that the hydrocarbyl group is saturated.  
It is further preferred that the hydrocarbyl group be a  
straight chain. The heteroatom hydrocarbyl group may  
contain more than one oxygen atom, sulfur atom or  
15 combination thereof in the principal chain, although  
oxygen is the preferred heteroatom. It is more  
preferred, however, that the principal chain contain  
only one oxygen or sulfur. Furthermore, it is preferred  
that the heteroatom not be  $\alpha$  to the acyl group or be on  
the omega position of the chain. It is most preferred  
20 that there may be only one heteroatom in the principal  
chain and that the heteroatom is oxygen.

Another preferred value of  $R_1$  and  $R_2$ , when  
defined as a heteroatom fatty acid acyl group, is the 1-  
substituted alkyl fatty acid acyl groups containing 3-  
25 25, carbon atoms and the substituents are hydroxy, halo,  
lower alkoxy, mercapto or alkylthio. In a more  
preferred embodiment, the substituent is hydroxy or  
halo, preferably bromo or chloro. In the most preferred  
embodiment, the hydrocarbyl chain contains 13-15 carbon  
30 atoms and most preferably 13-14 carbon atoms and is 1-  
substituted hydroxy or halo.

- 1           Of course, various combinations and  
permutations are possible, as described below, in which  
R<sub>1</sub> and R<sub>2</sub> are independently heteroatom alkyl group, 1-  
substituted alkyl group or heteroatom 1-substituted  
5 alkyl group. These various combinations and  
permutations are contemplated to be within the scope of  
the present invention.

Preferred embodiments of the present invention  
are directed to compounds wherein R is as defined  
10 hereinabove, one of R<sub>1</sub> and R<sub>2</sub> is hydrogen, alkyl fatty  
acid acyl group having 4-26 carbon atoms, or alkyl  
heteroatom fatty acid acyl group having 13-14 carbon  
atoms, and the other is independently a heteroatom  
hydrocarbyl fatty acid group containing 13-14 carbon  
15 atoms containing at least one oxygen or sulfur.

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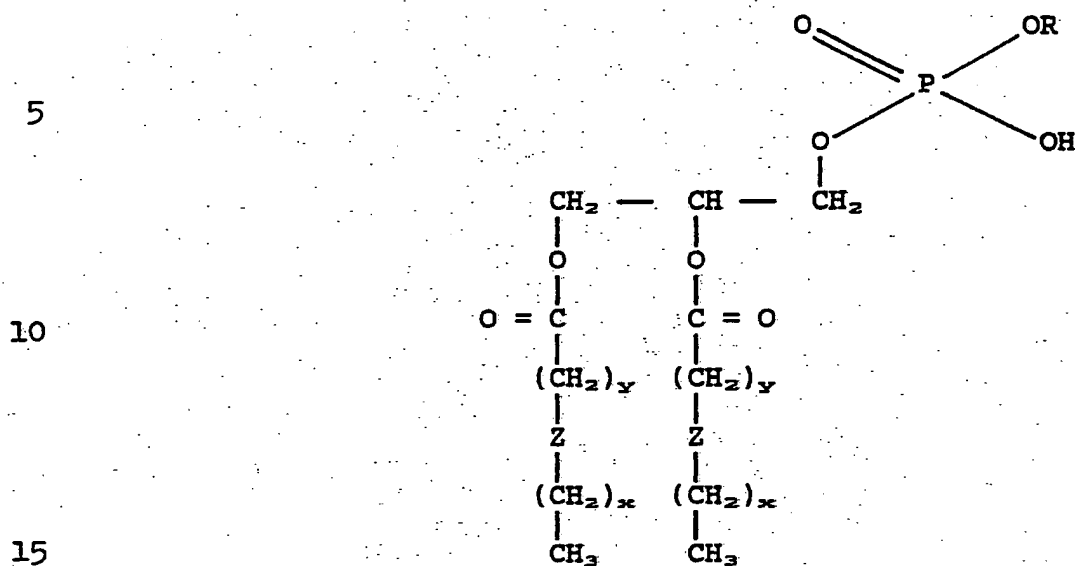
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1 In a further embodiment, the present invention  
 5 contemplates a phospholipid drug of the formula:



## VIII

wherein R is as defined hereinabove;

Z is oxygen or sulfur;

each x is independently 0 to 13;

20 each y is independently 1 to 13; and

x + y = 11-15 and most preferably 11.

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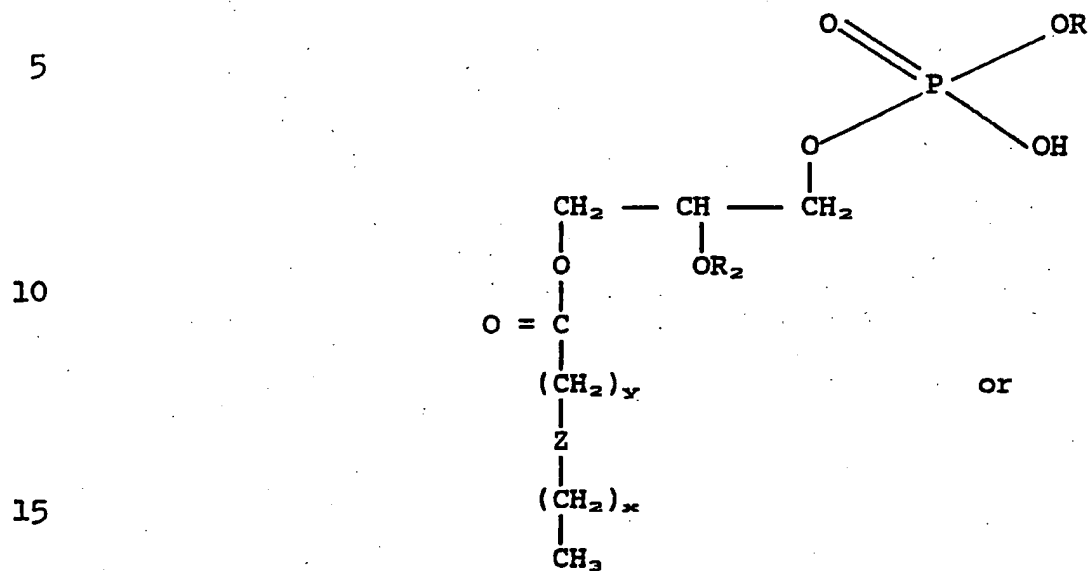
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1           The present invention also contemplates a  
 phospholipid drug of the formulae:



IX

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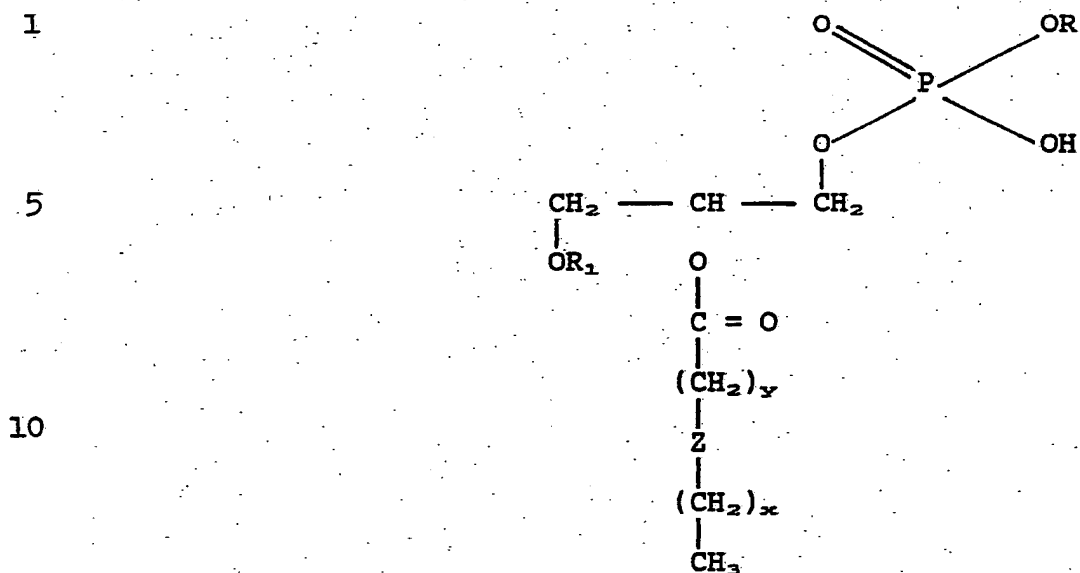
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wherein R is as defined hereinabove;

$R_2$  is hydrogen or alkyl fatty acid acyl group having 4-26 carbon atoms, and more preferably 4-8 or 14 carbon atoms,

Z is oxygen or sulfur;

each x is independently 0-13;

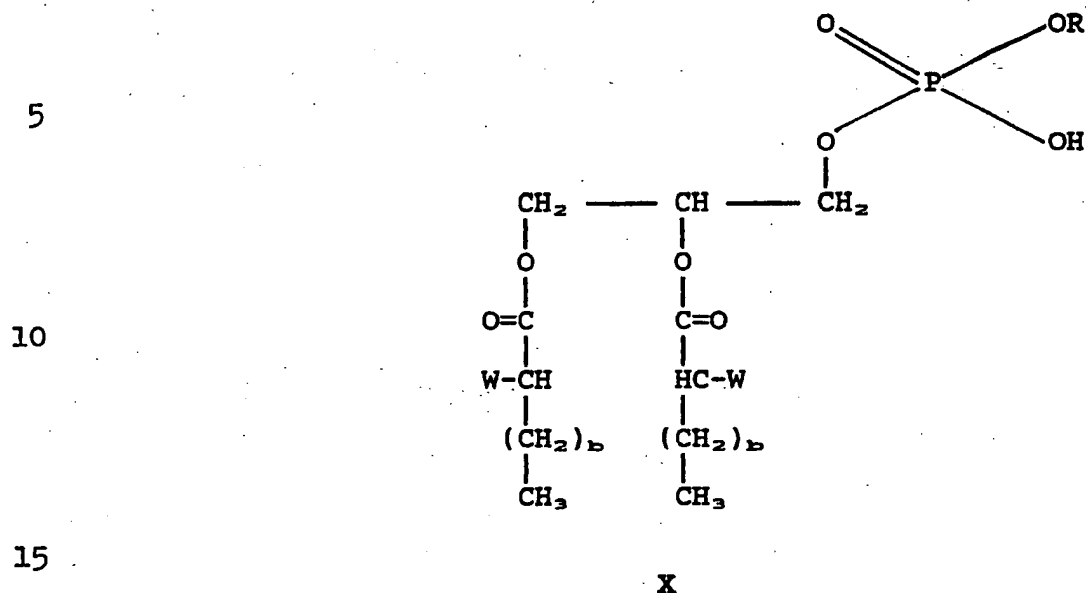
each y is independently 1-13; and

$x + y = 11-13$ , and most preferably 11.

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1 In a further embodiment, the present invention  
 5 relates to a phospholipid drug of the formula:



wherein W is halo, hydroxy, alkoxy, mercapto or  
 20 alkylthio; and R is as defined hereinabove and  
 b is 11-13 and most preferably 11.

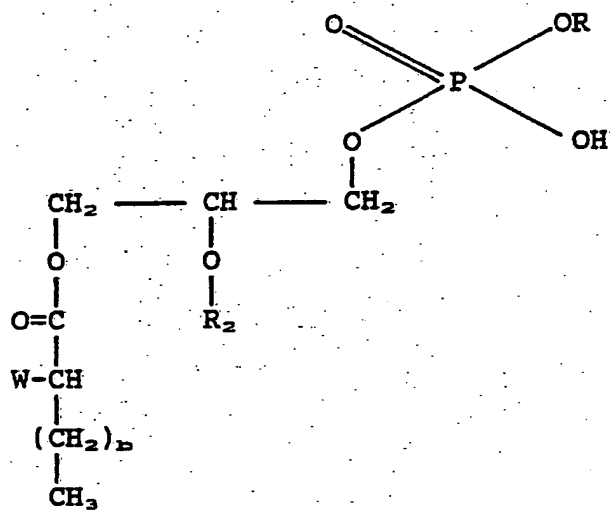
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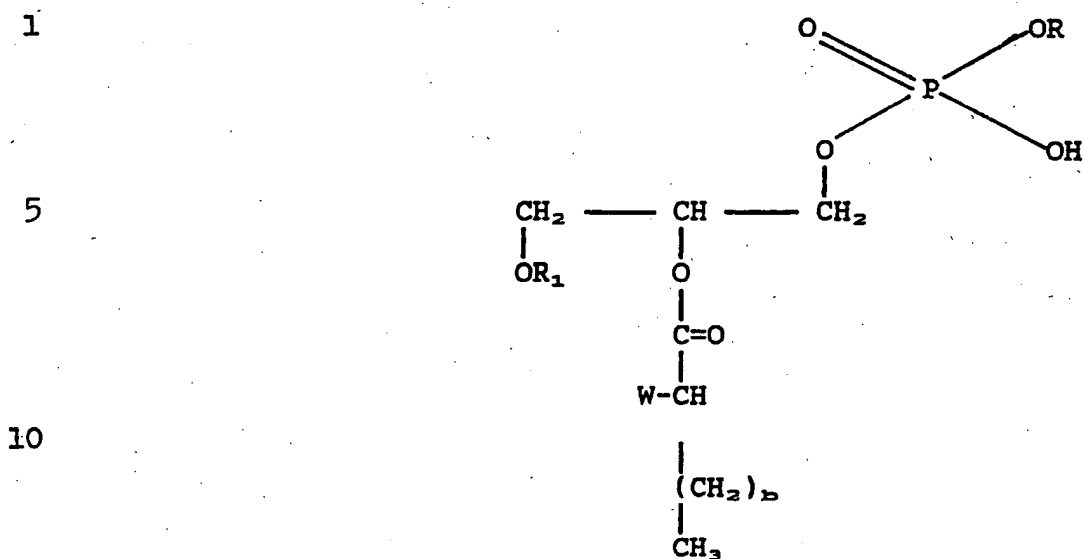
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Another preferred embodiment of the present invention relates to a phospholipid drug of the formulae:



**XI** or



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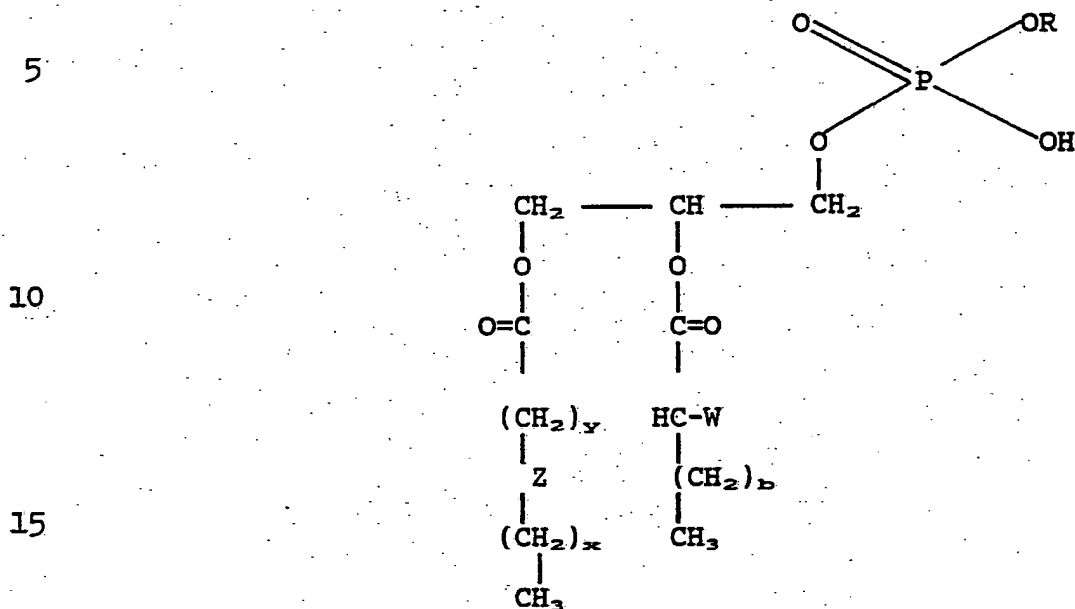
wherein R is as defined hereinabove;

R<sub>1</sub> and R<sub>2</sub> are independently hydrogen or alkyl fatty acid acyl group having 4-26 carbon atoms and more preferably 4-8 or 14 carbon atoms,

b is 11-13, and most preferably 11

and W is selected from halo, alkoxy, mercapto or alkylthio.

1 In a still further embodiment, the present  
invention relates to a phospholipid drug of the formula:



XIII

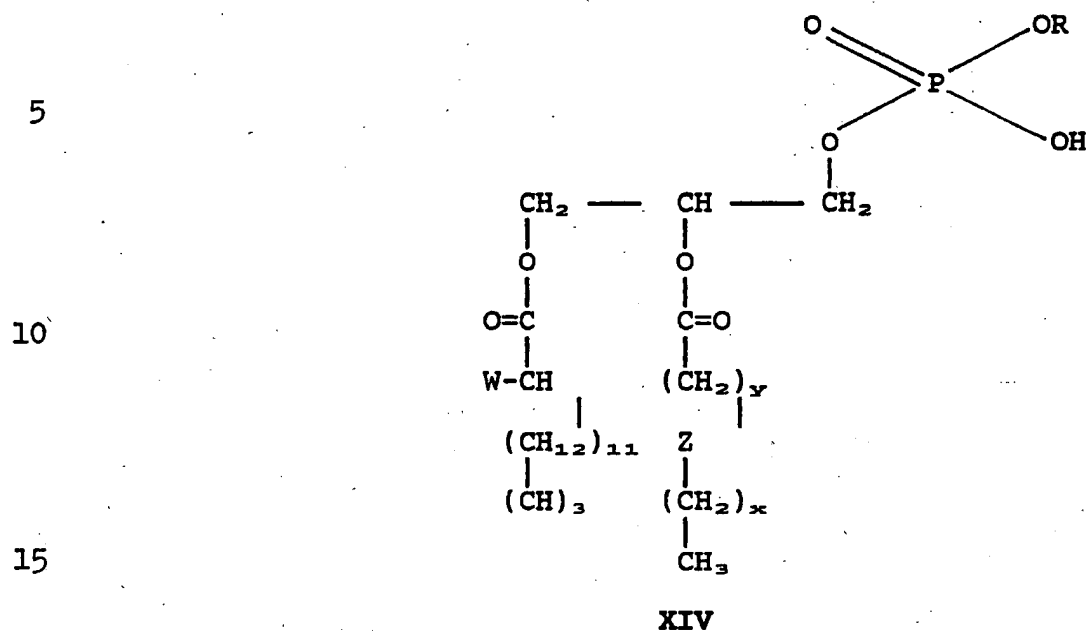
20 wherein R, Z, W, x, y and b are as defined hereinabove.

25

30

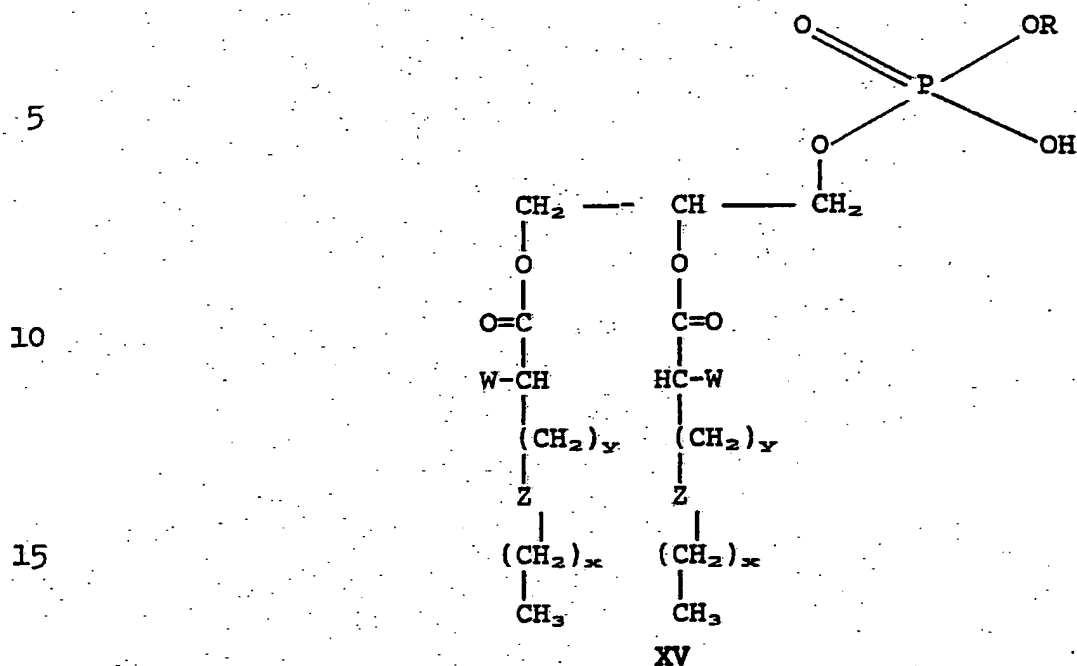
35

1 Another embodiment of the present invention  
 5 contemplates a phospholipid drug of the formula:



wherein R, Z, W, x and y are as defined hereinabove.

1            In a further embodiment, the present invention  
contemplates a phospholipid drug of the formula:



wherein R, Z, W, x, y are each as defined hereinabove. The compounds described in Formulae VIII to IX also contemplate the basic salts, as defined herein.

In the various embodiments described herein, it is preferred that R is

H;

$$\text{CH}_2 \text{ CH}_2 - \overset{\oplus}{\text{N}}(\text{CH}_3)_3;$$
$$-\text{CH}_2 \text{ CH}_2 - \text{NH}_2,$$
$$\begin{array}{c} -\text{CH}_2 - \text{CH} - \text{CH}_2 - \text{OH} \text{ or} \\ | \\ \text{OH} \end{array}$$

30 
$$-CH_2 - \underset{\substack{| \\ NH_2}}{CH} - COOH.$$

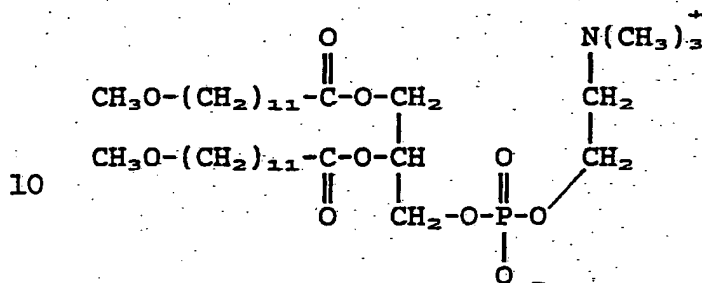
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1 In another embodiment, the present invention  
contemplates the compound 1,2-(di-12-methoxydodecanoyl)-  
sn-3-phosphatidylcholine (L-AC2) represented by the  
formula:

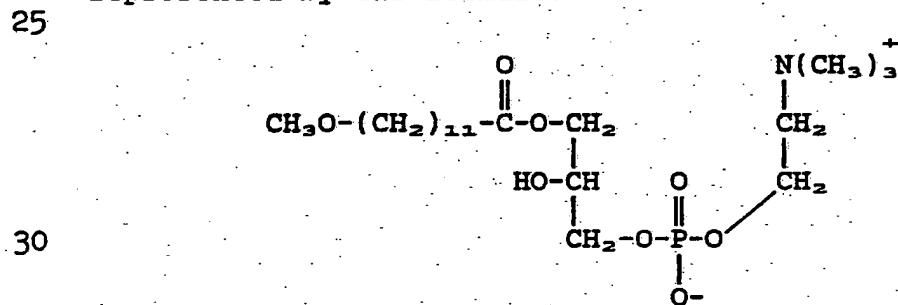
5 AC2



15 Compound AC2 contains the fatty acid 12MO bonded to both  
the sn-1 and sn-2 positions of the glycerol backbone.  
Furthermore, the configuration at the sn-2 carbon of the  
glycerol backbone is in the L configuration.

20 The present invention also contemplates the D-  
AC2 molecule, wherein the configuration at the sn-2  
carbon of the glycerol backbone is in the D  
configuration.

25 In still another embodiment, the present  
invention contemplates the compound: 1-(12-  
methoxydodecanoyl)-sn-3-glycerophosphatidylcholine  
represented by the formula:



35

1 hereinafter referred to as the "lysolipid analogs". The  
lysolipid analog contains the fatty acid 12MO bonded to  
the sn-1 position and hydrogen bonded at the sn-2 posi-  
tion of the glycerol backbone. Alternatively, the  
5 lysolipid analogs may contain the heteroatom fatty acid,  
(e.g., 12MO) bonded to the sn-2 position and the  
hydrogen bonded to the sn-1 position. Again, both the  
D- and L-stereoisomers contemplated by the present  
invention.

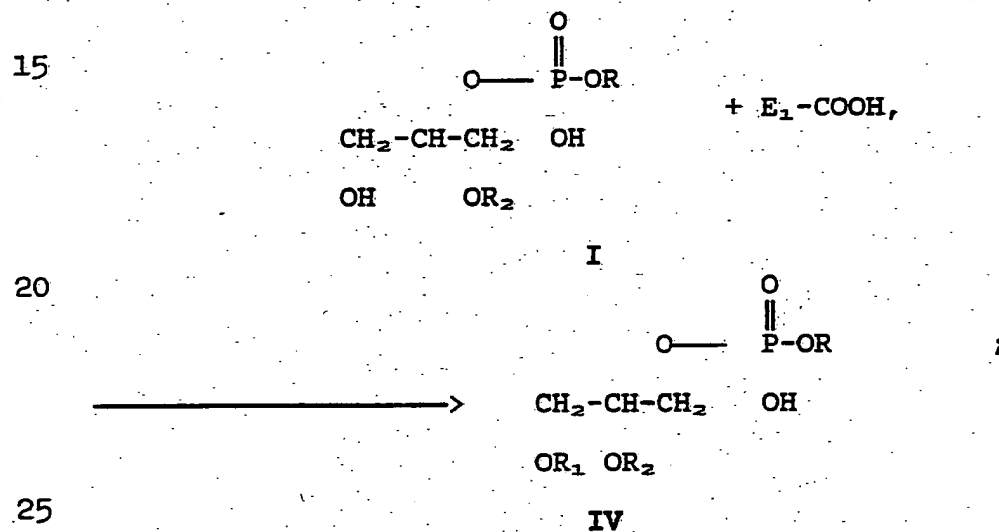
10 Compounds of the present invention can be  
prepared in accordance with art- recognized techniques.  
Exemplary procedures are described below.

The carboxy group on the heteroatom fatty acid  
is activated by standard methods for acylation and is  
15 reacted with the hydroxy group on the glycerol backbone  
under esterification conditions, known to one skilled in  
the art. The reaction may be run in inert solvents that  
will dissolve both reagents or it may be run in a  
biphasic solvent. Examples include DMSO, crown ethers  
20 and the like. The reaction is run at temperatures  
facilitating acylation. These temperatures may range  
from room temperature to the reflux temperature of the  
solvent, although it is preferred that the reaction is  
run at about room temperature or slightly above.  
25 Furthermore, the reaction may be run under reduced  
pressure, such as under vacuum.

Alternatively, the reaction may be run by  
first converting the acid to an acylating derivative,  
such as the acid halide (e.g., acid chloride, acid  
30 bromide) or anhydride, under reaction conditions known  
to one skilled in the art. The acylated derivative is  
then reacted with the hydroxy group on the glycerol

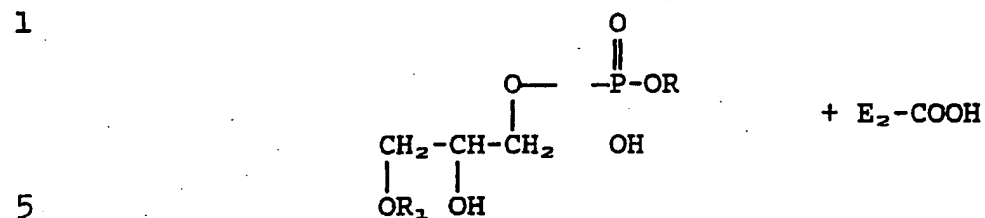
- 1 backbone of the glycerol phospholipid under  
 esterification conditions as described hereinabove. In  
 other words, the reaction may be run in an inert solvent  
 that will dissolve both reagents or it may be run in a  
 5 two-phase solvent system. The reaction is run at  
 temperatures facilitating acylation. These temperatures  
 may range from room temperature to the reflux  
 temperature of the solvent, although it is preferred  
 that the reaction is run at about room temperature or  
 10 slightly above. Further, the reaction may be run under  
 reduced pressure.

The reactions described hereinabove can be  
 schematically represented as follows:

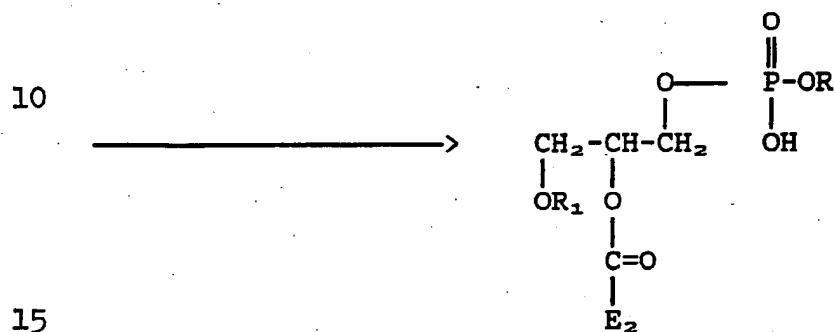


wherein  $\text{E}_1 - \text{C} = \text{R}_1$  and is a heteroatom fatty acid acyl  
 group;  $\text{E}_1 - \text{COOH}$  is a heteroatom fatty acid

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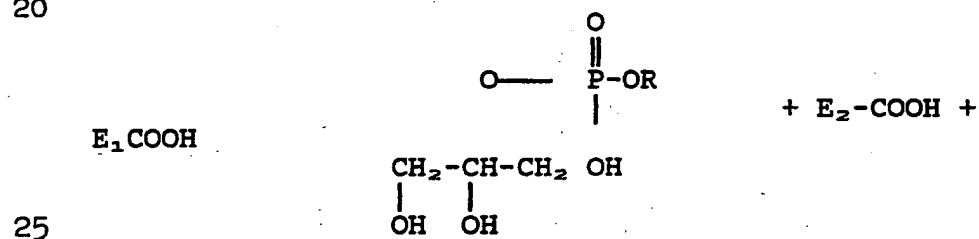
II



V

wherein  $\text{E}_2\text{C}=\text{O}$  is  $\text{R}_2$  and is a heteroatom fatty acid acyl group; and  $\text{E}_2\text{COOH}$  is a heteroatom fatty acid

20



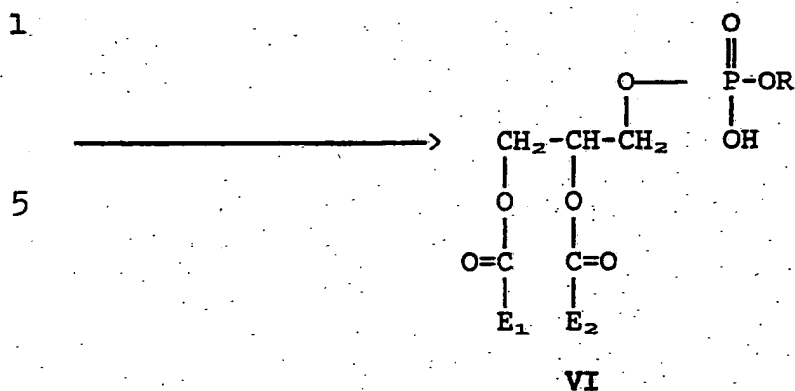
III

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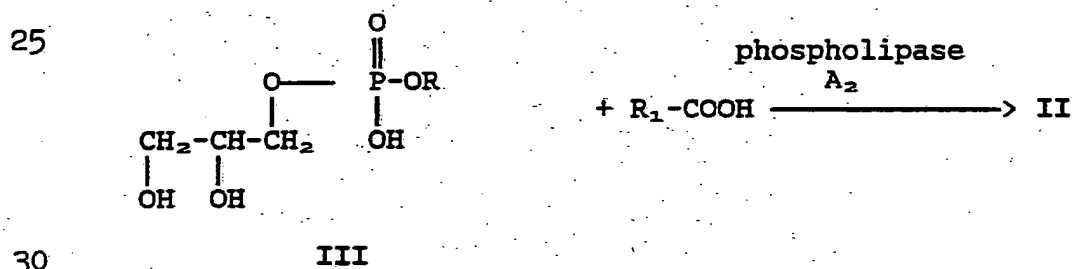
10 wherein  $\text{C} - \text{E}_1$  is  $\text{R}_1$  and  $\text{C} - \text{E}_2$  is  $\text{R}_2$ , and  $\text{E}_1 \text{ COOH}$

and  $\text{E}_2 \text{ COOH}$  are defined hereinabove.

15 In the above schemes,  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}$  are as defined hereinabove and  $\text{E}_1 \text{ COOH}$  and  $\text{E}_2 \text{ COOH}$  are the heteroatom fatty acids, as defined herein.

Compounds of Formula III are either available commercially or can be prepared by art recognized methods.

20 Compounds of Formula II wherein  $\text{R}_1$  is other than hydrogen can be prepared from compounds of Formula III as follows:

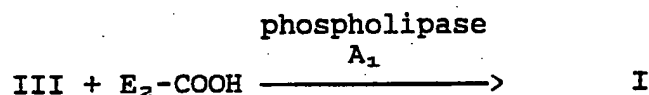


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1           Acylation of III with an excess of acylating derivative of  $R_1$ -COOH (e.g., the acid halide, anhydride or acid) under esterification conditions will produce the diacylated compounds. The esterification conditions 5 are similar to those described hereinabove. Hydrolysis of the acylated compound with phospholipase  $A_2$  will produce the compound of Formula II.

          Similarly, compounds of Formula I wherein  $R_2$  is other than hydrogen can be prepared from compounds of 10 Formula III as follows:

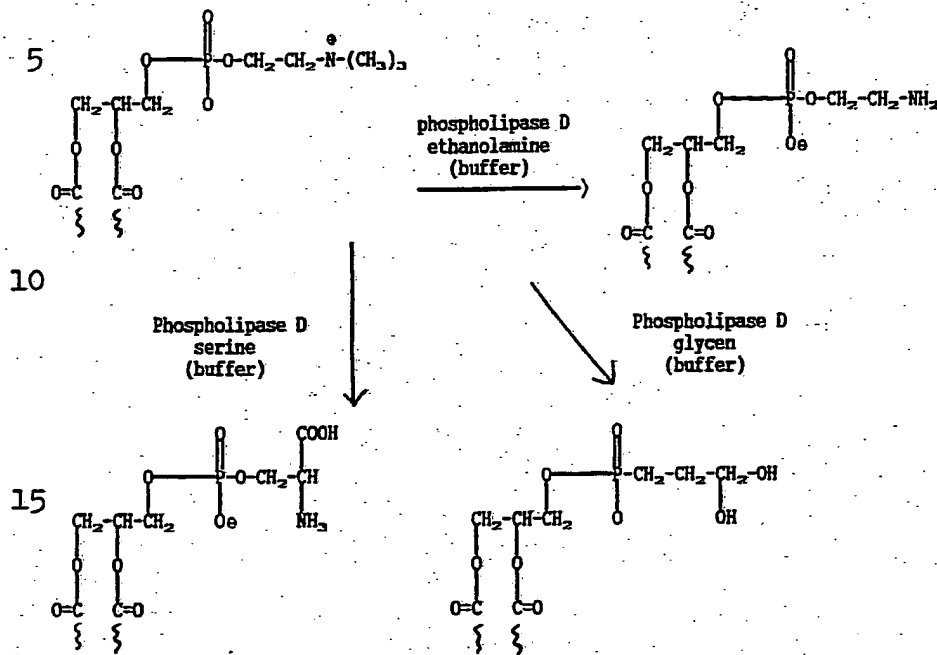


15           Acylating III with an excess of an acylating derivative of  $E_2$ -COOH (e.g., the acid halide, anhydride or acid) under esterification conditions as described hereinabove will produce the diacylated compounds. Hydrolysis of the diacylated compounds with 20 phospholipase  $A_1$  will produce the compound of Formula I.

          In both cases described hereinabove, the acid halide can be prepared from the corresponding acid with thionyl chloride or bromide. Similarly, the anhydride can be prepared from the corresponding acid by reacting 25 the acid with a dehydrating agent, such as  $P_2O_5$  or dicyclohexyl-carbodiimide. Alternatively, the anhydride can be prepared by reacting the acid halide with the corresponding salt of the acid.

          Furthermore, diacylated analogs containing 30 either a glycerol (PG), serine (PS) or ethanolamine (PE) headgroup can be synthesized by transphosphatidylation

1 using phospholipase D treatment of diacylated  
phosphatidyl choline analogs, as described hereinbelow.



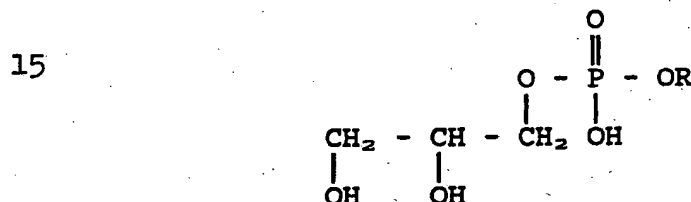
20 wherein represents the remainder of the fatty acid  
chain. The transphosphatidyl transfer with phospholipase  
D is effective with both the L- and D- stereoisomers of the  
glycerophospholipid. Cleavage with phospholipase A<sub>1</sub> or  
phospholipase A<sub>2</sub> of the L-isomer will produce the 1-  
25 hydroxy or 2-hydroxy analog, respectively.

The acylating derivative of the heteroatom  
fatty acid can be prepared in accordance with art-  
recognized procedures. For example, the acid chloride  
can be prepared by reacting the fatty acid with thionyl  
chloride. The anhydride can be prepared by reacting the  
30 fatty acid containing a free carboxy group with a  
dehydrating agent, such as P<sub>2</sub>O<sub>5</sub> or



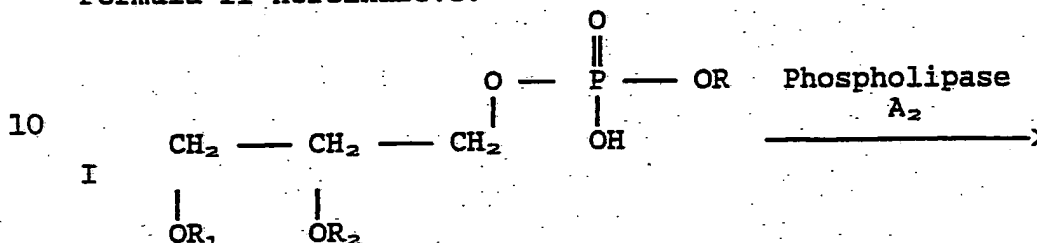
ldicyclohexylcarbodiimide, acetic anhydride, trifluoroacetic anhydride, methoxyacetylene and the like. Alternately, the anhydride can be prepared by treating the acid halide (such as acid chloride) of the fatty acid with the acid salt of the drug.

Alternatively, and especially in the case of the phospholipid drugs of myristic acid derivatives, the phospholipid drug can be prepared using immobilized artificial membranes (IAM), as described in Markovich, *et al.* in Anal Chem., 1991, 63, 1851-1860, the contents of which are incorporated herein by reference. The procedure will be described in more detail hereinbelow. Generally, the glycerol phospholipid of the formula:



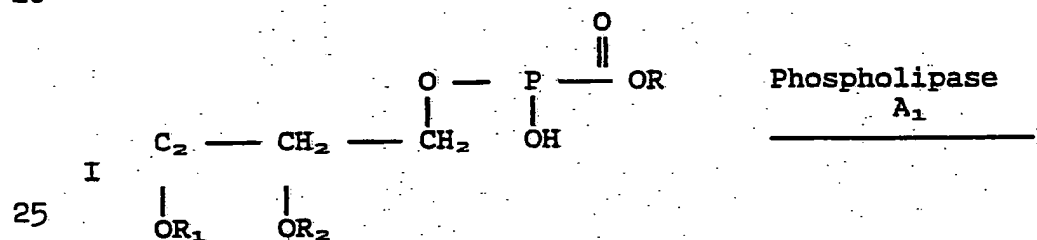
20(hereinafter referred to as GP) wherein R is as defined hereinabove (1 mMol) is solubilized in MeOH (0.5-2.0ml) and is adsorbed onto an IAM packing material, prepared as described hereinbelow by dropwise addition of the methanolic-PC solution. The MeOH was allowed to 25evaporate after the IAM surface was completely loaded with GP. The IAM/GP solid material was dried overnight in a vacuum at 45°C. After drying, the IAM/GP powdered was suspended in dry chloroform containing the dried acylating derivative of the drug (acid halide, 30anhydride, and the like) and dried equivalent of a catalyst, such as dimethylaminopyridine and the like.

- 1 The monoacylated phospholipid compounds of the  
 present invention (lyso form) can also be prepared from  
 the diacylated phospholipids by using the appropriate  
 phospholipase. For example, phospholipase A<sub>2</sub>  
 5 selectively hydrolyzes the ester at the sn<sub>2</sub> position of  
 the glycerol backbone to form a compound having the  
 Formula II hereinabove:



- 15 In the above scheme, R, R<sub>1</sub> and R<sub>2</sub> are as  
 defined hereinabove, except that R<sub>1</sub> and R<sub>2</sub> are not  
 hydrogen.

- Similarly, the other lyso form having Formula  
 I can be prepared from the diacylated phospholipid by  
 using phospholipase A<sub>1</sub>, which selectively hydrolyzes the  
 20 ester at the sn<sub>1</sub> position of the glycerol backbone:



A

- 30 Finally, the compound of Formula IV can be  
 prepared by hydrolysis of I with phospholipase A<sub>2</sub>,  
 hydrolysis of II with phospholipase A<sub>1</sub> or hydrolysis of  
 A with phospholipase A<sub>1</sub> and phospholipase A<sub>2</sub> in either

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1 order. It is to be noted that in the above schemes, R,  
R<sub>1</sub> and R<sub>2</sub> are as defined hereinabove, except that R<sub>1</sub> and  
R<sub>2</sub> are not hydrogen.

5 In the acylation reactions described above, E<sub>1</sub>  
COOH or E<sub>2</sub> COOH may be unsubstituted or substituted. If  
substituted, it is preferred that the substitution is on  
the  $\alpha$  carbon (the carbon atom adjacent to the carboxy  
group). Further, the preferred substituents are  
hydroxy, lower alkoxy, mercapto or alkyl thio.

10 These compounds can be prepared from art  
recognized techniques. For example, the  $\alpha$ -hydroxy  
compound can be prepared from the corresponding  $\alpha$ -halo  
carboxylic acid by reacting the latter with base (OH<sup>-</sup>)  
under substitution reaction conditions. Furthermore,  
15 the mercapto compound can be prepared from the  
corresponding  $\alpha$ -halo carboxylic acid by reacting the  
latter compound with HS<sup>-</sup> under substitution reaction  
conditions, while the  $\alpha$ -alkylthio carboxylic acid can be  
prepared from the corresponding  $\alpha$ -halo carboxylic acid  
20 by reacting the latter with lower alkylthiolate under  
substitution reaction conditions. The  $\alpha$ -lower alkoxy  
derivative can be prepared by reacting the  $\alpha$ -halo  
carboxylic acid with lower alkoxide under Williamson  
reaction conditions.

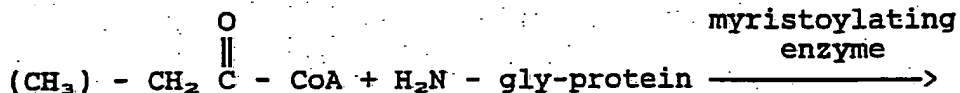
25 The  $\alpha$ -halo carboxylic acid can be prepared by  
reacting E<sub>1</sub> COOH (or E<sub>2</sub> COOH) with phosphorous and  
halogen (preferably Cl<sub>2</sub> or Br<sub>2</sub>) or phosphorus trihalide  
under Hell-Volhard-Zelinsky reaction condition as  
described hereinbelow on Pages 53-54. Alternatively,  
30 the  $\alpha$ -halo carboxylic acid can be prepared from the  
malonic acid ester synthesis described hereinbelow.

1 It is to be understood that in some of the  
 reactions described hereinabove, it may be necessary to  
 employ protecting groups on reactive functional groups,  
 such as hydroxy, that may be present. The protecting  
 5 groups to be employed are obvious to one skilled in the  
 art. Examples of various protecting groups can be found  
 in "Protective Groups in Organic Synthesis" by T.W.  
 Green, John Wiley and Son, 1981, which is incorporated  
 herein by reference.

10 In the reactions described hereinabove, the  
 various products can be separated and purified by art  
 recognized techniques known to one skilled in the art,  
 such as flash chromatography or HPLC.

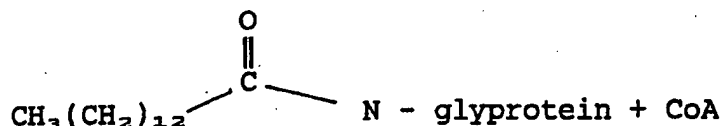
The phospholipid compounds resulting from the  
 15 above reactions are used to treat diseases caused by  
 retroviruses, such as AIDS and ARCS in animals,  
 especially mammals, by administering to said animal an  
 effective amount of the compound to treat said diseased  
 state. The present invention is directed to those  
 20 compounds as well as the use of the compounds in  
 treating diseases caused by retroviruses. Without  
 wishing to be bound, it is believed that the compounds  
 of the present invention interfere with protein  
 myristoylation, a reaction which is necessary for HIV  
 25 infections.

The HIV-1 genome encodes for two myristoylated  
 proteins: p<sup>17gag</sup> and p<sup>27pol</sup>. In situ myristoylation of  
 these proteins is critical for the establishment and  
 maintenance of HIV infection. The myristoylation  
 30 reaction can be represented as follows:



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N-myristoyltransferase (NMT) is the enzyme that cotranslationally transfers the myristoyl group to endogenous cellular and viral proteins. It is believed that the compounds of the present invention exhibit inhibitory activity against viruses that produce

10 myristoylated proteins.

Interference with protein myristoylation has been a drug target site for inhibiting HIV replication. It has been reported that heteroatom analogs of myristic acid containing oxygen or sulfur substituted for alkyl methylene groups exhibit activity against HIV replication in infected cells. European Patent Application 415,902 alleges that oxy and thio substituted fatty acid analog substrates of myristoylating enzymes in which a methylene group at carbon position 4 to 13 is replaced by an oxygen or sulfur can be used to treat retroviral infections. It has also been reported that metabolic activation of 2-substituted derivatives of myristic acid inhibits myristoyl CoA: Protein N-myristoyltransferase. See Paige, et al., Biochemistry 1990, 29, 10566-10573.

25

However, the present inventors have discovered that the efficacy of these compounds have been significantly enhanced by acylating these molecules to the glycerol backbone of a phospholipid in accordance with the present invention, thus generating new phospholipid drugs. More particularly, the fatty acid analogs of myristic acid are acylated to one or both of

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1 the hydroxy groups of the glycerophospholipid, i.e. the  
non-polar end. The acylation at the non-polar end of  
the phospholipid significantly influences the ability to  
inhibit HIV replication in macrophages and T cells and  
5 also alters the toxicity of the fatty acid analogs.  
Additionally, these acylated phospholipids may be  
sensitive to phospholipases A1 and A2, thereby providing  
a specific cleavage mechanism for the acyl group(s)  
containing the biologically active fatty acids, once the  
10 product is transported into the cell.

The present compounds can be formulated with  
suitable pharmaceutically acceptable carriers into unit  
dosage form and can be administered orally,  
transdermally parenterally or rectally. The active  
15 compound may be orally administered, for example, with  
an inert diluent or with an assimilable edible carrier,  
or it may be enclosed in hard or soft shell gelatin  
capsule, or it may be compressed into tablets, or it may  
be incorporated directly with the food of the diet. For  
20 oral therapeutic administration, the active compound may  
be incorporated with excipients and used in the form of  
ingestible tablets, buccal tablets, troches, capsules,  
elixirs, suspensions, syrups, wafers and the like. Such  
compositions and preparations should contain at least 1%  
25 of active compound. The percentage of the compositions  
and preparations may, of course, be varied and may  
conveniently be between about 5 to about 80% of the  
weight of the unit. The amount of active compound in  
such therapeutically useful compositions is such that a  
30 suitable dosage will be obtained. Preferred composi-  
tions or preparations according to the present invention  
are prepared so that an oral dosage unit form contains a

1 pharmaceutically effective amount which can be  
determined by the physician. For example, the oral  
dosage unit form may contain between about 0.5 and 1000  
mg of active compound.

5           The tablets, troches, pills, capsules and the  
like may also contain the following: A binder such as  
gum tragacanth, acacia, corn starch or gelatin;  
excipients such as dicalcium phosphate; a disintegrating  
agent such as corn starch, potato starch, alginic acid  
10 and the like; a lubricant such as magnesium stearate;  
and a sweetening agent such as sucrose, lactose or  
saccharin may be added or a flavoring agent such a  
peppermint, oil of wintergreen or cherry flavoring.  
When the dosage unit form is a capsule, it may contain,  
15 in addition to materials of the above type, a liquid  
carrier. Various other materials may be present as  
coatings or to otherwise modify the physical form of the  
dosage unit. For instance, tablets, pills or capsules  
may be coated with shellac, sugar or both. A syrup or  
20 elixir may contain the active compound, sucrose as a  
sweetening agent, methyl and propylparabens as  
preservatives, a dye and flavoring such as cherry or  
orange flavor. Of course, any material used in  
preparing any dosage unit form should be  
25 pharmaceutically pure and substantially non-toxic in the  
amounts employed. In addition, the active compound may  
be incorporated into sustained-release preparations and  
formulations.

          The active compound may also be administered  
30 parenterally or intraperitoneally. Dispersions can also  
be prepared in glycerol, liquid polyethylene glycols,  
and mixtures thereof and in oils. Under ordinary

- 1 conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for  
5 injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy  
10 syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for  
15 example, water, ethanol, polyol (for example, glycerol, propylene, glycol, and liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by  
20 the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol,  
25 sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying  
30 absorption, for example, aluminum monostearate and gelatin.



1 Sterile injectable solutions are prepared by  
incorporating the active compound in the required amount  
in the appropriate solvent with various of the other  
ingredients enumerated above, as required, followed by  
5 filtered sterilization. Generally, dispersions are  
prepared by incorporating the various sterilized active  
ingredient into a sterile vehicle which contains the  
basic dispersion medium and the required other  
ingredients from those enumerated above. In the case of  
10 sterile powders for the preparation of sterile in-  
jectable solutions, the preferred methods of preparation  
are vacuum drying and the freeze-drying technique which  
yield a powder of the active ingredient plus any  
additional desired ingredient from previously sterile-  
15 filtered solution thereof.

As used herein, "pharmaceutically acceptable  
carrier" includes any and all solvents, dispersion  
media, coatings, antibacterial and antifungal agents,  
isotonic and absorption delaying agents and the like.  
20 The use of such media and agents for pharmaceutical  
active substances is well known in the art. Except  
insofar as any conventional media or agent is  
incompatible with the active ingredient, its use in the  
therapeutic compositions is contemplated. Supplementary  
25 active ingredients can also be incorporated into the  
compositions.

It is especially advantageous to formulate  
parenteral compositions in unit dosage form for ease of  
administration and uniformity of dosage unit. Dosage  
30 form as used herein refers to physically discrete units  
suited as unitary dosages for the mammalian subjects to  
be treated; each unit containing a predetermined

1 quantity of active material calculated to produce the  
desired therapeutic effect in association with the  
required pharmaceutical carrier. The specification for  
the novel dosage unit forms of the invention are  
5 dictated by and directly dependent on (a) the unique  
characteristics of the active material and the  
particular therapeutic effect to be achieved, and (b)  
the limitations inherent in the art of compounding such  
an active material for the treatment of disease in  
10 living subjects having a diseased condition in which  
bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded  
for convenient and effective administration in effective  
amounts with a suitable pharmaceutically acceptable  
15 carrier in dosage unit form as hereinbefore disclosed.  
The physician can determine the amount of drug to be  
utilized. A unit dosage form can, for example, contain  
the principal active compound in amounts ranging from  
about 0.5 to about 1000 mg. In the case of compositions  
20 containing supplementary active ingredients, the dosages  
are determined by reference to the usual dose and manner  
of administration of the said ingredients.

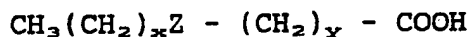
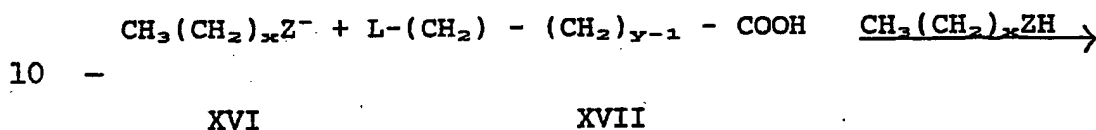
The general descriptions above are  
specifically illustrated hereinbelow with representative  
25 examples, AC-1, AC-2 and the lysoanalogs thereof. The  
following application of the generalizations hereinabove  
are provided solely for illustrative purposes. The  
invention is not to be limited in any way by the  
exemplification hereinbelow. The AC1 and AC2 and the  
30 lyso compound are the most preferred embodiments of the  
present invention and can be prepared by art recognized

- 1 synthetic procedures. Exemplary schemes are as outlined below.

Heteroatom-fatty acids

- 5 The general synthetic scheme for synthesizing hetero-atom-fatty-acids is outlined in EPA 0,415,902, which is incorporated herein by reference.

Synthetic scheme:



- 15 wherein x, y and Z are as defined hereinabove and L is a leaving group, such as halo, OTS, OMS and the like. A base having Formula XVI is reacted with a carboxylic acid of Formula XVII under Williamson-like conditions in  $\text{CH}_3(\text{CH}_2)_x\text{ZH}$ . The reaction is run at effective
- 20 temperatures, which may range from room temperature up to reflux temperatures, although it is preferred that the reaction be carried out under reflux temperatures. The following example illustrates the formation of the heteroatom fatty acids.

- 25 The general synthetic scheme for obtaining 12 MO is outlined below. A flame dried 300-ml round bottom flask was cooled before 8.4 g (0.030 mol) of 12-bromododecanoic acid was mixed with 6.5 g (0.120 mol) sodium methoxide in 200 ml of absolute methanol. The
- 30 yellow solution was refluxed at 85°C for 16-20 hours under a nitrogen atmosphere. After refluxing, the mixture was allowed to cool and the solvent was removed

1 by rotoevaporation. After removing most of the solvent,  
approximately 2-4 milliliters of residue remained and  
was extracted by the addition of ethyl acetate 100 ml,  
ether 50 ml and H<sub>2</sub>O 50 ml. Prior to acidification, the  
5 organic layer was clear and the top aqueous layer was  
yellow. This organic/aqueous mixture was acidified to  
pH 3 with 1 N HCl causing the organic layer to yellow  
and the aqueous layer to become clear. The aqueous and  
organic layers were separated and the aqueous layer was  
10 extracted twice with 30-50 ml of ethyl acetate. The  
organic extracts were pooled and washed once with 50 ml  
of H<sub>2</sub>O. The organic layer was dried using anhydrous  
Na<sub>2</sub>SO<sub>4</sub> and filtered. After removing the solvent by  
rotoevaporation, the residue was heated (50°C) under  
15 vacuum for 5 hours to remove trace organic solvents.  
TLC analysis using ethyl acetate:hexanes:formic acid  
88:9:3, gave  $R_f = 0.25$ . Typical yields range from about  
80-95%.

20 Hetero-atom-fatty-acid anhydrides

The corresponding anhydride of the heteroatom  
fatty acid is formed by coupling the heteroatom fatty  
acid with a dehydrating agent, such as  
dicyclohexylcarbodiimide, as illustrated by the  
25 exemplary procedure hereinbelow.

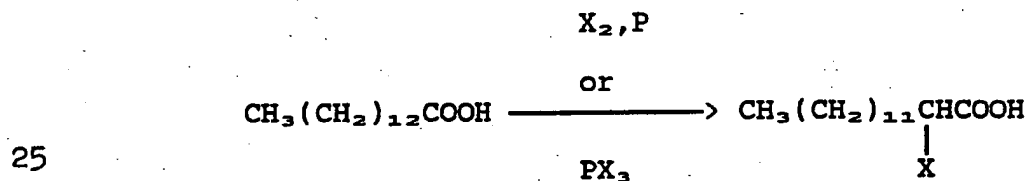
The anhydride was prepared in a flame dried  
50-ml round bottom flask containing 5.4 g (0.024 mol) of  
12-methoxydodecanoic acid completely dissolved in 20 ml  
of dry THF under a nitrogen atmosphere. After adding  
30 DCC 2.40 g (0.012 mol) dropwise over 5 minutes, the  
reaction was complete in under 25 minutes as monitored  
by the disappearance of the DCC imine vibration band

1 (centered at  $2100\text{ cm}^{-1}$ ). Preliminary studies showed if  
 solid DCC was added to the reaction mixture then several  
 side products were found by TLC. Consequently DCC was  
 melted, weighed into a flame dried beaker, and diluted  
 5 with 5 ml of dry THF. Dicyclohexylurea (DCU) precipi-  
 tates within the first few minutes of DCC addition. DCU  
 was removed by paper filtration (Whatman #1). The  
 solvent was removed by rotoevaporation and placed under  
 a heated vacuum ( $50^{\circ}\text{C}$ ) for 12 hours. FTIR and TLC  
 10 analysis (ethyl acetate:hexanes:formic acid 88:9:3)  
 revealed no DCU or DCC in the final product. Typical  
 yields were 90-95%.

#### 15 Substituted myristic acid analogs

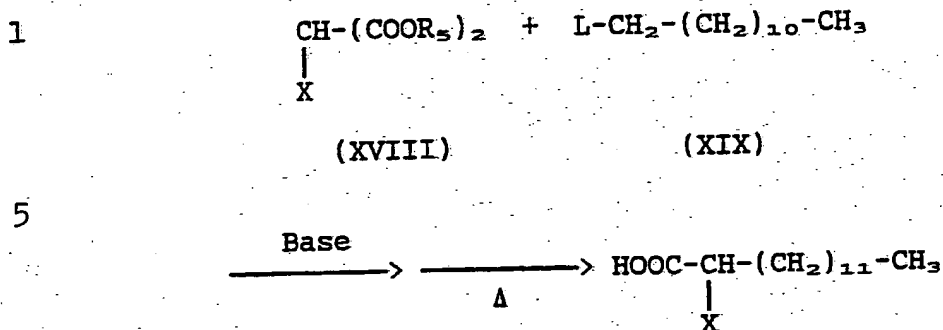
The synthesis of the substituted myristic acid  
 is also prepared by art recognized techniques. Although  
 the reaction substrate described hereinbelow is myristic  
 acid, the following examples are exemplary and are  
 20 applicable to fatty acids in general.

The fatty acid can be halogenated as follows:



wherein X is halo, e.g., bromo or chloro. The fatty  
 acid is halogenated with phosphorus in the presence of  
 halogen, or with  $\text{PX}_3$  under Hell-Volhard-Zelinsky  
 30 reaction conditions to form the  $\alpha$ -halogenated product.  
 Alternatively, the acid may be formed in two steps using  
 a variation of the malonic ester synthesis:

35



In the above scheme, X is halo, R<sub>5</sub> is lower alkyl, such as methyl, ethyl and the like, and L is a better leaving group than X, such as OTS, OMS and the like. For example, if X is F, then L may be OTS, OMS, Br, I and the like.

As described hereinabove, the halo malonic acid ester (XVIII) is reacted with a strong base to remove the acidic hydrogen on the α-carbon. The resulting anion is then reacted with an alkyl halide (XIX) and forms the coupled diester. The reaction is run in an inert solvent, such as dimethyl formamide, and preferably under anhydrous conditions. The resulting product is then heated at temperatures effective for decarboxylation to form the final product.

The reaction is further exemplified by the following example.

The general synthetic scheme for synthesizing substituted myristic acid analogs is similar to the synthesis of 2-fluoromyristic acid which can be synthesized as follows: To a suspension of 0.32 g (11.2 mmol) of an 80% oil dispersion of NaH in 8 mL of dry DMF was added dropwise 2 g (11.2 mmol) of diethyl fluoromalonate under argon. The suspension was then stirred for 4.5 h after which time, 2.79 g (11.2 mmol)

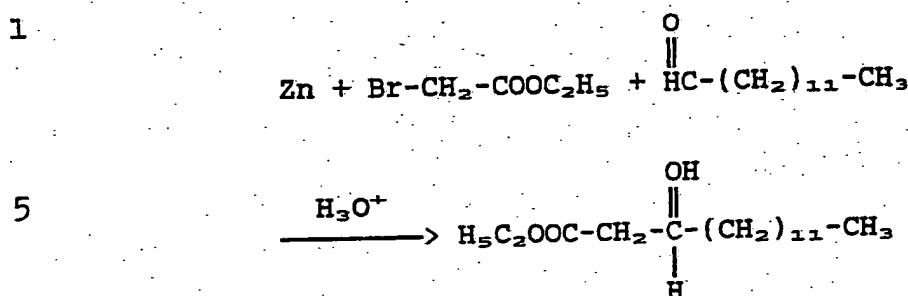
1 of 1-bromododecane was added and the solution was heated  
at 90°C for 18 h. The yellow suspension was then poured  
into 10 mL of water and extracted with ether (2 x 15  
mL). The combined ether layers were washed, dried and  
5 evaporated. A yellow oil resulted, which was used for  
the next reaction without further purification. A  
mixture of 3.8 g of the crude  
diethyldodecylfluoromalonate (8), 30 mL of 6 N HCl, and  
50 mL of dioxane was refluxed for 72 h. After cooling,  
10 the yellow solution was dissolved in 100 mL of petroleum  
ether (bp 40-60°C). The organic layer was separated and  
washed with water (3 x 50 mL) and 10% KOH (2 x 250 mL).  
The combined aqueous layers were acidified to pH 1.0  
with concentrated HCl and extracted with ether (3 x 100  
15 mL). The ether layer was dried, filtered and evaporated  
to dryness to yield a green solid. The solid was  
decolorized with activated carbon and recrystallized  
from petroleum ether (bp 40-60°C) to give 1.501 g (54%  
overall yield) of 2-fluoromyristic acid (2) as white  
20 needles.

2-bromomyristic acid was purchased from  
Aldrich and 2-hydroxymyristic acid was purchased from  
Fluka.

A beta hydroxy acid can be formed by reacting  
25 a  $\beta$ -halo ester with an aldehyde in the presence of zinc  
under Reformatsky reaction conditions in an inert  
solvent, such as toluene or DMF.

30

35



The resulting product is then hydrolyzed to form the corresponding acid.

10 The  $\beta$ -hydroxy compound can then be converted to the corresponding halide by art recognized techniques, such as reaction with thionyl bromide or chloride, phosphorous trihalide, ( $\text{SO}_2\text{Cl}_2$ ,  $\text{SO}_2\text{Br}_2$ ,  $\text{PCl}_3$ ,  $\text{PI}_3$ ) and the like.

15 The corresponding anhydrides of the substituted fatty acid analog can be prepared by coupling the substituted fatty acid analog prepared hereinabove with a dehydrating agent, such as decyclohexylcarbodiimide, as illustrated by the  
20 exemplary procedures hereinabove.

### Phospholipids

The phospholipids are prepared by art recognized techniques by reacting an acylating  
25 derivative of the fatty acid, such as the fatty acid anhydride, with the glycerol phosphate of the formula:



wherein R is as defined hereinabove, under esterification conditions. This reaction is illustrated



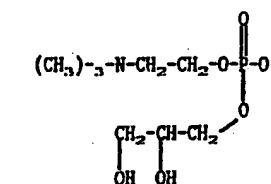
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1 hereinbelow. Although the reaction is illustrated using the heteroatom fatty acid to form the heteroatom fatty acid phospholipid, the reactions described hereinbelow are applicable using the substituted fatty acid analogs 5 to form the phospholipid containing the substituted fatty acid analogs.

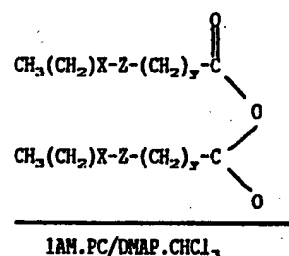
The chemical reaction for the solid phase adsorption synthesis of L-AC2 is given in Rxn. 1. The synthetic route for obtaining the anhydride used in Rxn. 101 is given above.

Rxn.1

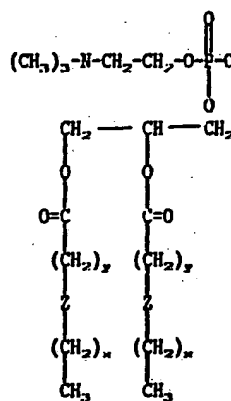
15 Solid Phase Synthesis



Glycerophosphocholine (GPC)



20



25

Diacylated phosphatidylcholine analogs

X = 0-11  
 Y = 0-11  
 x+y = 11  
 Z = oxygen or sulfur

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1           Phospholipids are not difficult to synthesize,  
but the present novel-synthetic-method overcomes several  
experimental inconveniences associated with phospholipid  
synthesis. During the synthesis of diacylated  
5   phospholipids, an experimental inconvenience involves  
the insolubility of glycerophosphocholine (GPC) in  
common organic solvents. Although GPC is soluble in  
dimethylsulfoxide (DMSO), the use of DMSO requires  
vacuum distillation and in addition, DMSO makes the  
10   purification of phospholipids more difficult. The  
inventors have developed a novel method for synthesizing  
molecules when all reactants are not soluble in the same  
organic solvent. The method involves using a chro-  
matographic surface to promote the interaction between  
15   one insoluble reactant and one soluble reactant. In  
other words, the insoluble-reactant is initially  
adsorbed to the chromatographic surface, and the  
soluble-reactant partitions into the chromatographic  
interface during the reaction. Partitioning of the  
20   soluble-reactant between the chromatographic surface and  
the reaction solvent permits the insoluble molecule,  
adsorbed at the interfacial region, to react with the  
soluble reactant. Although other chromatographic  
surfaces may be useful, IAM.PC chromatographic surfaces  
25   were utilized.

          The synthesis of AC2 demonstrates the solid-  
phase-synthetic procedure shown in Rxn. 1. GPC (250 mg,  
1 mMol) solubilized in MeOH (0.5 ml), was adsorbed to  
IAM.PC (200 mg) by dropwise addition of the methanolic-  
30   GPC solution; the MeOH was allowed to evaporate after  
the IAM.PC surface was completely loaded with GPC. The  
IAM.PC/GPC solid material was dried overnight in a

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1 vacuum oven at 45°C. After drying, the IAM.PC/GPC  
powder was then suspended in dry CHCl<sub>3</sub> containing 1  
equivalent of the appropriate anhydride per GPC alcohol,  
and 1 equivalent of catalyst (i.e.,  
5 dimethylaminopyridine denoted as DMAP). Both the  
anhydride and DMAP were dried by vacuum at 45°C. After  
6 hours, TLC confirmed the reaction was complete, and  
the phospholipid product was purified by acetone  
precipitation and/or silica chromatography. Normally  
10 the synthesis would have required 4-5 days per  
phospholipid and 2 equivalents of anhydride, but this  
method requires approximately 1-2 days and 1 equivalent  
of anhydride.

General structures of mono- and diacylated  
15 phospholipids whereby all alkyl chains are biologically  
active fatty acids are shown below. For both the mono-  
and diacylated phospholipids:  $x = 0-11$ ;  $y = 0-11$ , and  $x$   
 $+ y = 11$  for any given analog. "Z" denotes the hetero-  
atom and will be either oxygen or sulfur. The chemical  
20 reactions for the synthesis of each compound is also  
given below.

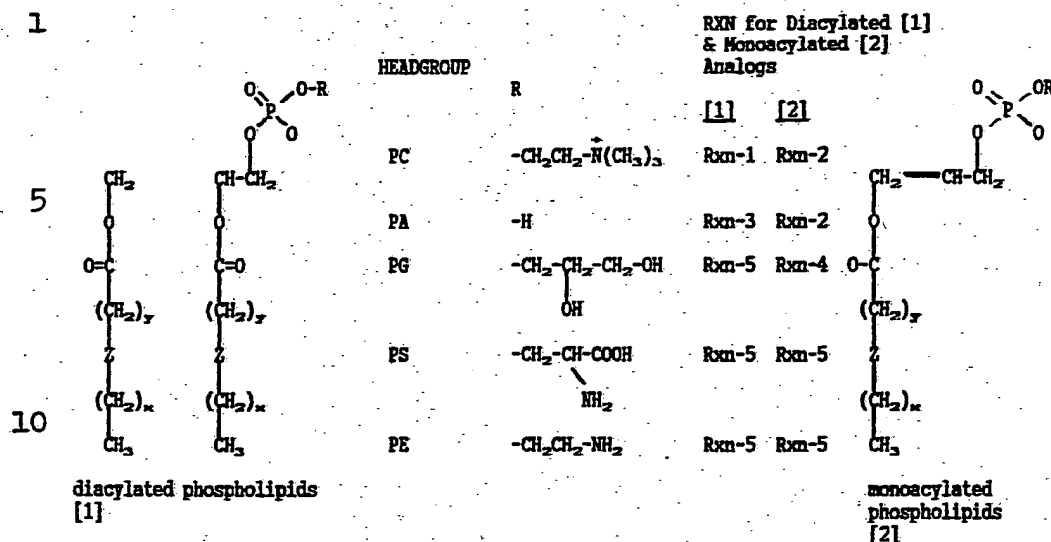
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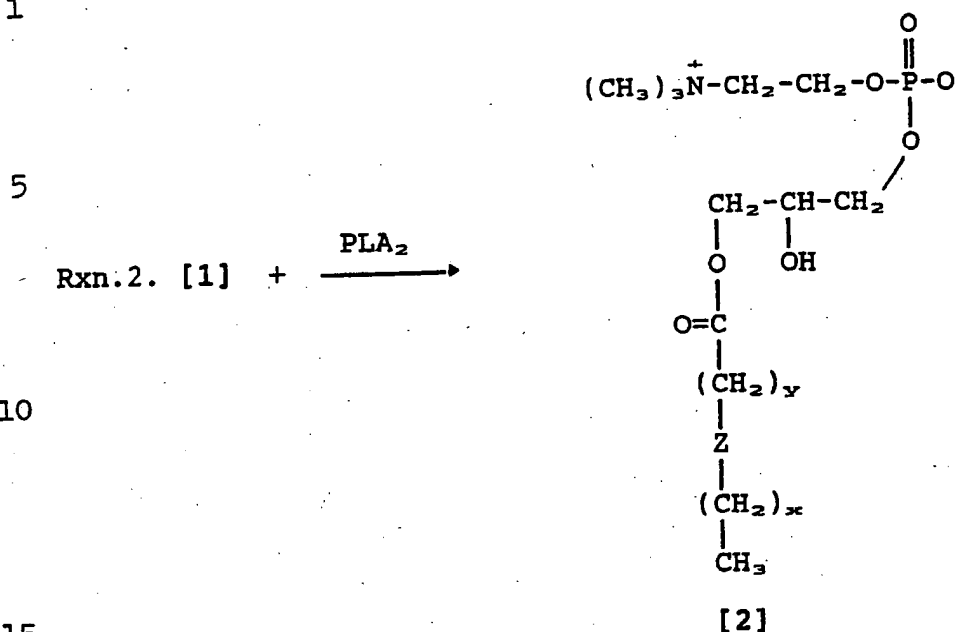
Chemical reactions 2-6 for the above synthetic routes are illustrated below. Briefly, diacylated analogs were prepared by a solid-phase adsorption method (see Rxn. 1 at Pages 54-55 of the application), monoacylated analogs can be prepared from phospholipase A2 treatment of the diacylated compounds, and diacylglycerol analog can be prepared from a separate reaction scheme (i.e., reaction 6). Reaction 6 involves first protecting glycidol epoxide using diphenylsilyl-(t)-butylchloride to form glycidol-tert-butyl diphenylsilyl ether. The epoxide ring is then opened with base, and the protected glycerol is diacylated with 12-methoxydodecanoyl anhydride. The final step in reaction 6 involves deprotecting the diacylglycerol sn-3 alcohol using n-butylammonium fluoride.

Diacylated phosphatidylcholine analogs containing oxygen or sulfur substituted for methylenes can be hydrolyzed with phospholipase A2 to obtain the corresponding lysolecithin analogs.

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Monoacylated Phosphatidylcholine

Diacylated phosphatidic acid analogs can be synthesized by reacting L-glycerol-3-phosphate with the appropriate anhydride on the surface of an immobilized artificial membrane particle. This reaction was described in detail in the discussion of reaction 1 using glycerophosphatidylcholine as starting material (see Reaction 3).

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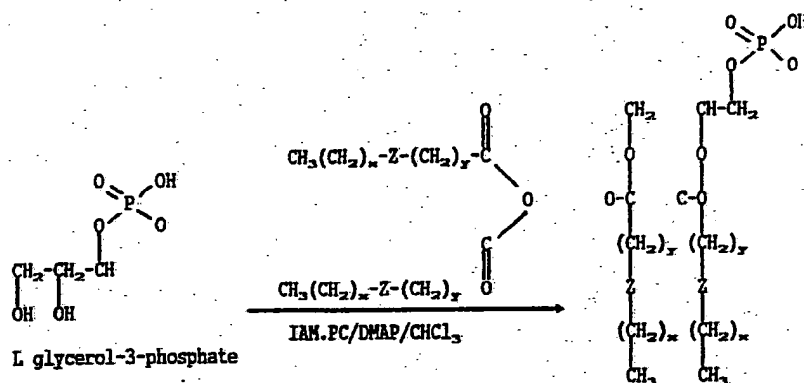
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Rxn.3.

Solid Phase

[3]



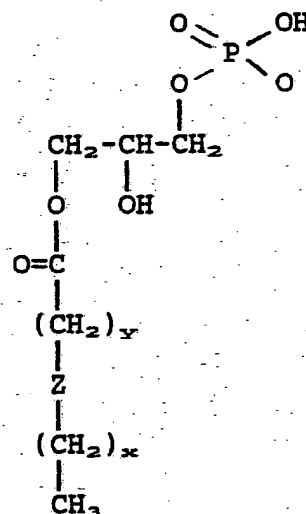
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Monoacylated analogs of phosphatidic acid can be obtained by phospholipase A<sub>2</sub> cleavage of the diacylated analogs denoted by [3] in reaction 3. This reaction is described hereinbelow:

15

20 Rxn.4

[3]

PLA<sub>2</sub>

[ 4 ]

Monoacylated phosphatidic acid

30

35

- 1                   Diacylated analogs containing either a  
glycerol (PG), serine (PS), or ethanolamine (PE)  
headgroup can be synthesized by transphosphatidylation  
using phospholipase D treatment of diacylated  
5   phosphatidylcholine analogs denoted as [1] in rxn. 5.  
The lysolipid analogs with these headgroups can then be  
obtained by further reaction with phospholipase A2  
cleavage.

10

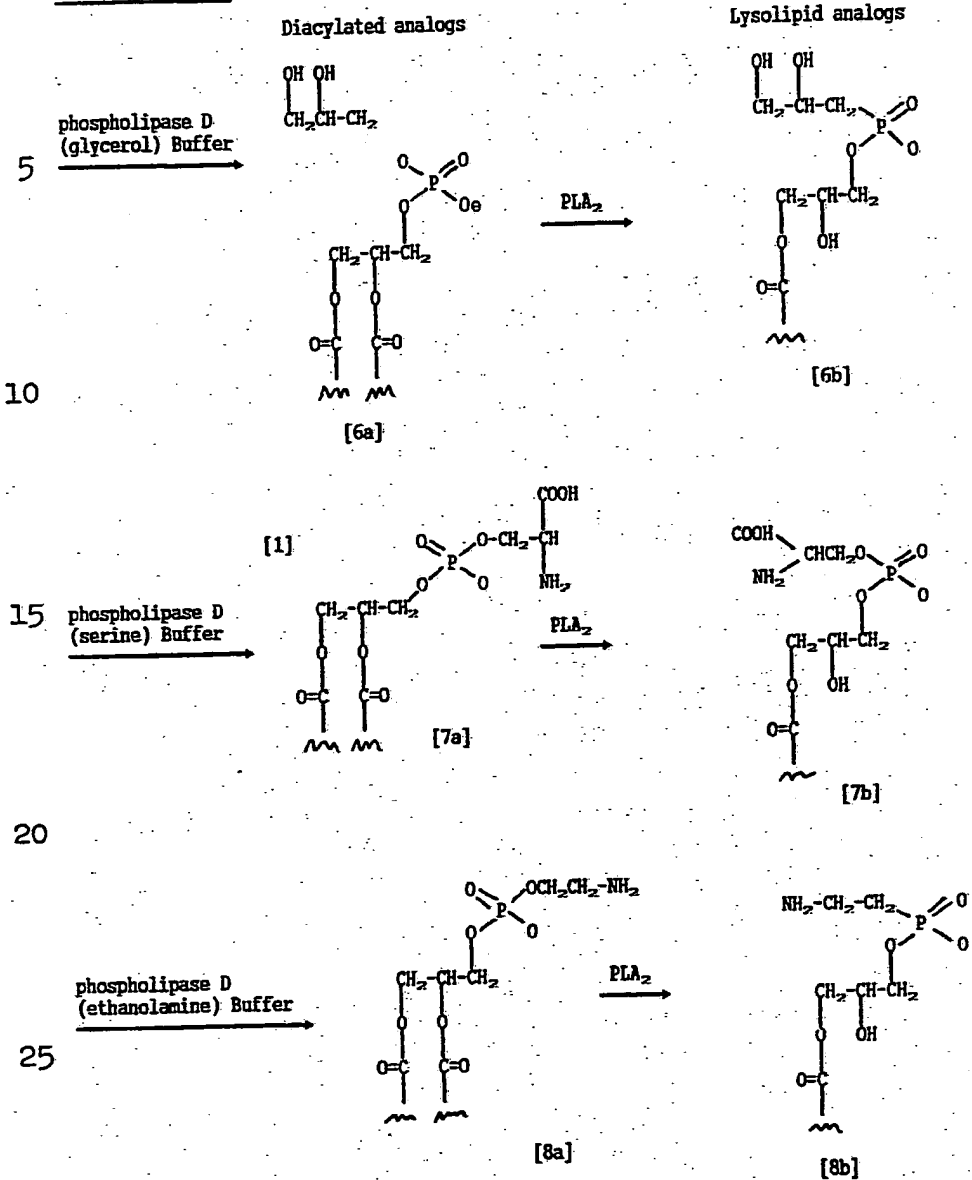
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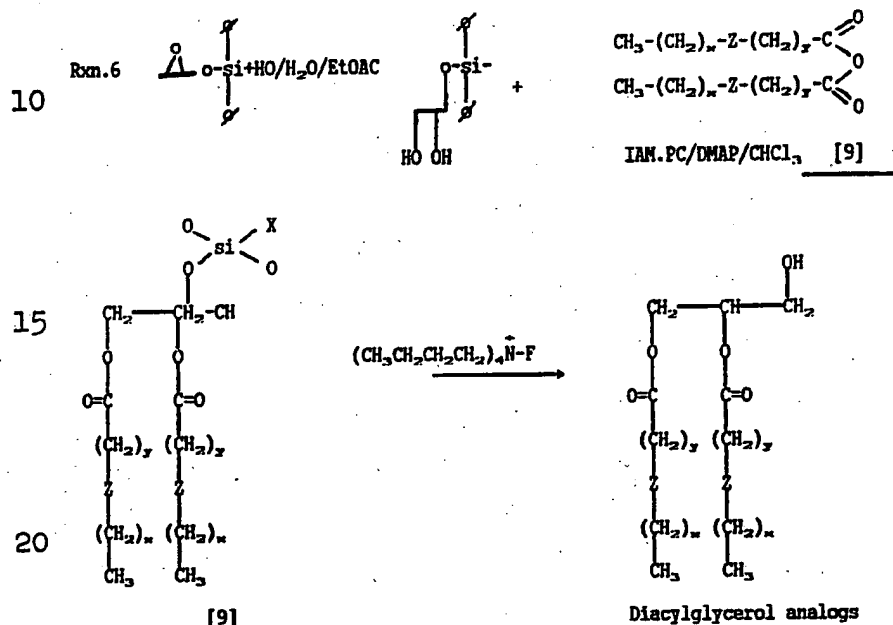
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1 REACTION 5



- 1 Diacylglycerol analogs containing two identical hetero-atom-fatty acids can be synthesized from (t)butyldi-phenylsilyl-O-glycidol. This reaction is performed by solid phase adsorption method employed in reaction 1 using glycerophosphatidylcholine as substrate, as shown below.



1

EXAMPLES

## GENERAL SYNTHESIS

Lecithins are 'diacylated' phospholipids and scheme 1 and scheme 2 shows the general synthetic pathway used to obtain anti-HIV lecithins containing the phosphatidyl-ethanolamine headgroup. Scheme 1 shows the general synthetic scheme used to prepare a lipid containing two 12-methoxy-dodecanoyl groups and a phosphatidylethanolamine headgroup; this lipid is denoted as di-12MOGPE. Briefly, L- $\alpha$ -GPE was reacted with Fmoc-NHS to form GPE-Fmoc in a mixed solvent system; this reaction protected the 1° amine of GPE. GPE-Fmoc was then acylated with 12-MO-anhydride in dry chloroform to form the diacylated product (di-12MOGPE-Fmoc). Di-12MOGPE was then obtained by removing Fmoc with piperidine. Scheme 2 shows the general synthetic scheme used to prepare the anti-HIV lecithin containing one 12-methoxydodecanoyl group and one saturated fatty acid. Briefly, monomyristoylphosphatidylethanolamine (MMPE) was reacted with Fmoc-NHS to form MMPE-Fmoc; this reaction protected the 1° amine of MMPE. MMPE-Fmoc was then acylated with 12-MO-anhydride in dry tetrahydrofuran to form the diacylated product 1-M-2-12MOGPE-Fmoc. 1-M-2-12 MOGPE was then obtained by removing the Fmoc group with piperidine.

Scheme 3 shows the general synthetic scheme used to prepare the anti-HIV lipid containing the phosphatidylcholine headgroup. Briefly, diacylated lecithins were prepared from both the L and D form of glycerophosphocholine (L- $\alpha$ -GPC and D- $\alpha$ -GPC) by acylation using 12-MO anhydride. The single chain analog of the L configuration was then prepared from phospholipase A2

35

1 cleavage of the diacylated product. For all reactions described above, acylation using the 12-MO-anhydride used 2 equivalents of anhydride per alcohol and 1.5 equivalents of catalysts (DMAP) per alcohol.

5

#### CHEMICAL AND SOLVENTS

Chemicals and solvents were used as received unless stated otherwise. 1-myristoyl-sn-glycero-3-phosphoethanolamine (MMPE) was purchased from Avanti 10 Polar Lipis Inc. (Birmingham, AL). L- $\alpha$ -glycerophosphoethanolamine (GPE) and N-(9-Fluorenylmethoxycarbonyloxy) succinimide (Fmoc-NHS) were purchased from Sigma Chemical Company. D- $\alpha$ -glycerophosphocholine was purchased from Biochemisches 15 Labor, Berne CH, Switzerland. Dimethylaminopyridine (DMAP) purchased from Aldrich was crystallized 2 times from ethyl ether. Dicyclohexylcarbodiimide (DCC) was purchased from Aldrich. Sodium bicarbonate ( $\text{NaHCO}_3$ ) was obtained from Fisher Scientific Chemical Company. 20 Analytical grade chloroform ( $\text{CHCl}_3$ ), methanol (MeOH), and tetrahydrofuran (THF) were obtained from Fischer Scientific.  $\text{H}_2\text{O}$  was double distilled from glass containers. Dry THF and Dry  $\text{CHCl}_3$  were prepared by distillation over calcium hydride. Calcium hydride was 25 purchased from Alpha Products, Danver, MA. 12-methoxydodecanoic acid 12MO was prepared as described hereinabove. 12-methoxyldodecanoyl anhydride (MO-anhydride) was prepared using DCC and purified by crystallization using ethylacetate. Piperidine was 30 obtained from Fisher Scientific Chemical Company. Ninhydrin and Phospray were purchased from Supelco Inc. Bellefonte, PA.

1           Thin layer chromatography (TLC) was used to  
monitor all reactions. Silica gel TLC plates were 60 F-  
254, 0.25 mm thickness (E. Merck, Darmstadt, FGR). Two  
TLC solvent systems were used: solvent system A  
5   contained  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$  65:25:5 V:V:V; solvent B  
contained  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{THF}$  64:34:7:30 V:V:V:V. TLC  
plates were sprayed with either Ninhydrin (Supelco Inc.  
Bellefonte, PA) to visualize amines or Phospray (Supelco  
Inc. Bellefonte, PA) to visualize phosphate. The extent  
10 of reaction was routinely quantified using a scanning  
densitometer (Shimadzu CS 9000) operating in the  
reflectance mode. TLC plates were sprayed with Phospray  
prior to scanning at 600 nm. Phospholipid standards  
were always included on the same TLC plate used for  
15 lipid quantification. Silica gel for flash  
chromatography was grade 60, 230-400 mesh and obtained  
from Aldrich Chemical Company. The solvent systems  
described hereinabove were also used to purify the  
heteroatom containing phospholipid drugs by flash  
20 chromatography.

L- $\alpha$ -glycerophosphoethanolamine-  
Fluorenylmethyloxycarbonyl (GPE-Fmoc).

GPE (93  $\mu\text{mole}$ , 20 mg), Fmoc-NHS (130  $\mu\text{mole}$ , 45  
25 mg) and  $\text{NaHCO}_3$  (288  $\mu\text{moles}$ , 24 mg) were transferred into  
a round bottom flask, and 10 mls of  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$   
(32:17:2 V:V:V) was immediately added to the flask. The  
reaction mixture was stirred at room temperature. GPE  
and Fmoc are soluble in this solvent system but  $\text{NaHCO}_3$   
30 is only slightly soluble. Based on TLC in solvent  
system A, quantitative yields are obtained in  
approximately three and half hours. The reaction

-59-

- 1 mixture was filtered to remove  $\text{NaHCO}_3$  (solid) and the  
filtrate was rotoevaporated to dryness. After  
rotoevaporation the residue was redissolved in  $\text{CHCl}_3$  (~1  
ml) and loaded on to dry silica gel loosely packed in a  
5 cylindrical glass frit filtration funnel (~5 g of silica  
per 1 g of reaction mixture). The unreacted Fmoc-NHS  
washed off with  $\text{CHCl}_3$  (10 mls), and NHS washed off with  
 $\text{CH}_3\text{OH}$  (10 mls). The product was then washed with  
 $\text{CHCl}_3/\text{CH}_3\text{OH}$  1:1 V:V (10 mls) to obtain pure GPE-Fmoc.  
10 GPE-Fmoc ( $R_f$  of 0.24) shows one spot on TLC plates  
developed in solvent system A. GPE-Fmoc is UV positive,  
Phospray positive, and Ninhydrin negative.

15 di-(12-methoxydodecanoyl)-sn-glycero-3-  
phosphoethanolamine-Fluorenylmethyloxycarbonyl (di-  
12MOGPE-Fmoc)

- GPE-Fmoc, MO-anhydride and DMAP were dried in  
a vacuum desiccator at  $40^\circ\text{C}$  for at least four hours  
before use. GPE-Fmoc (93  $\mu\text{mol}$ , 40 mg), MO-anhydride  
20 (410  $\mu\text{mol}$ , 180 mg) and DMAP (200  $\mu\text{mol}$ , 24 mg) were added  
to a flame dried round bottom flask and freshly  
distilled  $\text{CHCl}_3$  (10 mls) was added. The reaction  
mixture was under a  $\text{N}_2$  atmosphere and stirred at  $40^\circ\text{C}$ .  
After 20 hours, TLC in solvent system A confirmed that  
25 the reaction was virtually complete; the major product  
di-12MOGPE-Fmoc had an  $R_f$  of 0.46. Rotoevaporation of  
the reaction solvent left a dry residue which was  
redissolved in minimal  $\text{CHCl}_3$  (~1 ml). The  $\text{CHCl}_3$   
solubilized residue was loaded on to silica gel packed  
30 inside a cylindrical glass-frit filtration funnel (~5 g  
silica/g of residue). The unreacted MO-anhydride washed  
off the silica with  $\text{CHCl}_3$  (~200 ml); TLC in solvent

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1 system A was used to monitor MO-anhydride in the  
filtrate. We note that it is important to remove MO-  
anhydride from the crude product-mixture to avoid  
decreased retention times and coelution of the products  
5 and reactants during column chromatography. After the  
anhydride was removed,  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$  (60:35:5 V:V:V)  
(~50 mls) was used to wash off the reaction products.  
Rotoevaporation of the filtrate left a crude product-  
mixture. The product mixture was redissolved in a  
10 minimum volume of mobile phase  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$  (65:25:4  
V:V:V) and purified using flash chromatography (3 cm x  
21 cm column, ~2 mg of reaction mixture per 1 g of  
silica gel). Fractions eluting from the column (10  
ml/fraction) were analyzed by TLC in solvent system A.  
15 Fractions containing di-12MOGPE-Fmoc were pooled and the  
solvent removed by rotoevaporation. The pure product  
(di-12MOGPE-Fmoc) showed one UV positive TLC spot that  
was also Ninhydrin negative and Phospray positive. The  
yield was ~70%.

20

1,2 di-(12-methoxydodecanoyl)-sn-glycero-3-  
phosphoethanolamine (di-12MOGPE)

di-12 MOGPE-Fmoc was dissolved in dry  $\text{CHCl}_3$   
(20 mg/ml) at room temperature and piperidine was added  
25 [1:80 di-12MOGPE-Fmoc:piperidine]. Fmoc is completely  
removed in 2 hours but if twice the amount of piperidine  
is used, then 100% conversion occurs within 45 minutes.  
On TLC in solvent system A, di-12-MOGPE has an  $R_f$  of 0.3  
and is both Ninhydrin and Phospray positive but UV  
30 negative which indicates that the Fmoc group has been  
removed. The reaction solvent was removed by  
rotoevaporation and the crude residue dissolved in 1 ml

35

1 of solvent system A and purified by flash chromatography  
 (3 cm x 21 cm) using the same solvent system. Fractions  
 were collected (10 ml/fractions) and analyzed by TLC in  
 solvent system A. Fractions containing the product were  
 5 pooled and the solvent removed by rotoevaporation to  
 obtain pure di-12MOGPE. The final lecithin product (di-  
 12MOGPE) exhibited one spot on TLC in solvent system A  
 and was Ninhydrin positive, Phospray positive, and UV  
 negative.

10 FAB-MS:  $MH^+$  640.3. IR ( $CaF_2$ , neat)  $\nu_{as}$   $CH_2$   
 2917.4;  $\nu$   $CH_2$  2850.4;  $\nu$  C=O 1738.4;  $\delta_{as}$   $CH_2$  1454.0;  $\nu_{as}$   
 $PO_2$  1230.6;  $\nu$  C-O-C 1077.8;  $\nu_s$   $PO_2$  1027.9.  $^1H$  NMR (500  
 MHz,  $CDCl_3$ ) results:  $\delta$  5.18 ppm (br s, 1H, CH), 4.35  
 ppm (m, 1H,  $CH_2OP$ ), 4.11 ppm (m, 1H,  $CH_2OP$ ), 4.05 ppm  
 15 (br s, 2H,  $CH_2OP$ ), 3.90 ppm (br s, 2H,  $CH_2OCO$ ), 3.33 ppm  
 (t, 4H,  $OCH_2$ ), 3.30 ppm (s, 6H,  $OCH_3$ ), 3.12 ppm (br s,  
 2H,  $NCH_2$ ), 2.28 ppm (m, 4H  $CH_2COO$ ), 1.55 ppm (m, 8H  
 $CH_2CH_2COO$ ,  $CH_2CH_2OCH_3$ ), 1.25 ppm (br s, 28H,  $(CH_2)_7$ ).

20 1,3 di-(12-methoxydodecanoyl)-sn-glycero-2-  
phosphoethanolamine-Fluorenylmethyl-oxycarbonyl (1,3 di-  
12MOGPE-Fmoc)

Headgroup migration occurred during the  
 preparation of di-12MOGPE-Fmoc and the migration product  
 25 was 1,3 di-12 MOGPE-Fmoc. This migration product was  
 purified by flash chromatography as described above for  
 di-12MOGPE-Fmoc. 1,3 di-12MOGPE-Fmoc exhibited one spot  
 on TLC in solvent system A with an  $R_f = 0.4$ .

$^1H$  NMR (500 MHz,  $CDCl_3/CD_3OD$ ) results:  $\delta$  7.68  
 30 ppm (d, 2H, aromatic), 7.55 ppm (d, 2H, aromatic), 7.30  
 ppm (t, 2H, aromatic), 7.22 ppm (t, 2H, aromatic), 4.33  
 ppm (br s, 1H,  $CHOP$ ), 4.28 ppm (d, 2H,  $CH_2CHCC$ ), 4.10

- 1 ppm (t, 1H, CHCC), 4.15 ppm (m, 1H CH<sub>2</sub>OP), 3.90 ppm (m, 1H, CH<sub>2</sub>OP), 3.82 ppm (br s, 2H, CH<sub>2</sub>OP), 3.80 ppm (br s, 2H, CH<sub>2</sub>OCO), 3.42 ppm (br s, 2H, NCH<sub>2</sub>), 3.30 ppm (t, 4H, OCH<sub>2</sub>), 3.23 ppm (s, 6H OCH<sub>3</sub>), 2.15 ppm (t, 4H, CH<sub>2</sub>COO),  
5 1.50 ppm (m, 8H CH<sub>2</sub>CH<sub>2</sub>COO, CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>), 1.20 ppm (br s, 28H, (CH<sub>2</sub>)<sub>7</sub>).

1-myristoyl-sn-glycero-3-phosphoethanolamine-  
Fluorenylmethyl-oxycarbonyl (MMPE-Fmoc)

- 10 MMPE (9.88 mmoles, 4.2 g) and insoluble NaHCO<sub>3</sub> (29 mmoles, 2.4 g) were mixed in 50 mls of CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O 32:17:4 V:V:V for 2 minutes at room temperature prior to the addition of Fmoc-NHS (13.3 mmoles, 4.5 g). After 2-3 hours the reaction was  
15 virtually complete based on TLC in solvent system A. Without NaHCO<sub>3</sub> the yield was always 50-60% regardless of reaction conditions. The reaction was filtered through a fine glass-frit funnel to remove NaHCO<sub>3</sub> (solid) and the filtrate was rotoevaporated to obtain a residue.  
20 The dry residue (~9 g) was redissolved in minimum CHCl<sub>3</sub> (2-3 ml) and loaded on to dry silica loosely packed in a glass filtration funnel (~5 g of the silica gel per g of reaction mixture). Based on TLC in solvent system A, unreacted Fmoc-NHS washed off the silica with CHCl<sub>3</sub>  
25 (~100 mls/g-product). After removing Fmoc-NHS from the product-mixture, both the phospholipid-product and NHS byproduct coeluted using ~300 mls of CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (32:17:4 V:V:V). The mixed solvent containing the product was removed by rotoevaporation and the residue  
30 extracted with CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O 8:4:3 V:V:V to remove NHS and other impurities. The product remained in the organic phase during the extraction. During extraction



1 approximately 20% of the product was lost into the  
aqueous phase but was recovered by reextraction of the  
aqueous phase with fresh organic solvent. MMPE-Fmoc was  
an amorphous white solid after lyophilization from  
5 benzene. The pure MMPE-Fmoc shows one spot (Rf of 0.36)  
during TLC in solvent system A. MMPE-Fmoc is UV  
positive, Phospray positive and Ninhydrin negative.  
Product yields are 70-90% based on 2 reactions.

IR (CaF<sub>2</sub>, neat) results: OH 3336.0 (broad);  
10  $\nu_{\text{as}}$  CH<sub>3</sub> 3064.7;  $\nu_{\text{s}}$  CH<sub>3</sub> 2953.1,  $\nu_{\text{as}}$  CH<sub>2</sub> 2923.9;  $\nu_{\text{s}}$  CH<sub>2</sub>  
2852.9;  $\nu$ C=O 1721.7;  $\nu$ 1533.8;  $\delta_{\text{as}}$  CH<sub>2</sub> 1450.3;  $\nu_{\text{as}}$  PO<sub>2</sub>  
1236.2;  $\nu$ C-O-C 1108.5;  $\nu_{\text{s}}$  PO<sub>2</sub> 1069.0. <sup>1</sup>H NMR (500 MHz,  
CDCl<sub>3</sub>/CD<sub>3</sub>OD) results: 67.55 ppm (d, 2H, aromatic), 7.40  
ppm (d, 2H, aromatic), 7.18 ppm (t, 2H, aromatic), 7.10  
15 ppm (t, 2H, aromatic), 4.15 ppm (m, 1H, CH<sub>2</sub>OP), 3.95 ppm  
(t, 1H, CHCC), 3.90 ppm (d, 2H, CH<sub>2</sub>CHCC), 3.75 ppm (m,  
1H, CH<sub>2</sub>OP), 2.70 ppm (br s, 2H, CH<sub>2</sub>OP), 3.65 ppm (br s,  
2H, CH<sub>2</sub>OCO), 3.18 ppm (t, 2H, NCH<sub>2</sub>), 2.08 ppm (t, 2H,  
CH<sub>2</sub>COO), 1.35 ppm (m, 2H CH<sub>2</sub>CH<sub>2</sub>COO), 1.05 ppm (br s,  
20 20H, (CH<sub>2</sub>)<sub>10</sub>), 0.70 ppm (t, 3H, CH<sub>3</sub>).

1-myristoyl-2-[12-methoxydodecanoyl]-sn-glycero-3-  
phosphoethanol-amine-Fluorenylmethyloxycarbonyl (1-M-2-  
12 MOGPE-Fmoc).

25 MMPE-Fmoc, MO-anhydride and DMAP were dried in  
a 45°C vacuum desiccator for at least 4 hours. MMPE-  
Fmoc (154  $\mu$ mol, 100 mg) was dissolved in freshly  
distilled THF in a flame dried round bottom flask, and  
12-MO-anhydride (632  $\mu$ mole, 280 mg) and DMAP (460  $\mu$ mol,  
30 56 mg) were also dissolved in distilled THF but in a  
separate flask. Both flasks were heated to 45°C and  
after the reactants dissolved, MO-anhydride and DMAP

1were carefully transferred to the flask containing MMPE-  
Fmoc (20 mg reactant/ml solvent). The reaction mixture  
was purged with nitrogen and stirred. After 1 hour the  
reaction was cooled to room temperature and allowed to  
5react for another 14 hours. The solvent was removed by  
rotoevaporation and a minimum volume of  $\text{CHCl}_3$  (~1-2 ml)  
was used to dissolve the residue. Unreacted MO-  
anhydride was removed from the reaction mixture and the  
product was purified as described above for di-12MOGPE-  
10Fmoc with the minor modification that the mobile phase  
solvent was solvent system B. The purified product (1-  
M-2-12MOGPE-Fmoc) exhibited one spot ( $R_f = 0.48$ ) on TLC  
plates developed in solvent system B. 1-M-2-12MOGPE-  
Fmoc was Ninhydrin negative, phospray positive and UV  
15positive. The yield was ~92% based on 1 reaction.

1-myristoyl-2-[12-methoxydodecanoyl]-sn-glycero-3-  
phosphoethanolamine (1-M-2-12MOGPE)

Fmoc was removed from 1-M-2-12MOGPE-Fmoc by  
20piperidine and the lecithin product (1-M-2-12MOGPE)  
purified by flash chromatography as described above for  
di-12MOGPE. Similar to purification of di-12MOGPE, the  
purification of 1-M-2-12MOGPE was simple because Fmoc  
elutes at the solvent front whereas piperidine remains  
25at the origin when  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{THF}$  (64:34:7:30) is  
used as an isocratic solvent system during flash  
chromatography. The final product, 1-M-2-12 MOGPE,  
shows one spot on the TLC plates developed in solvent  
system A. 1-M-2-12-MOGPE is UV negative, and positive  
30when sprayed with either Ninhydrin or Phospray. The  
yield was ~84 % based on 1 reaction.

- 1 FAB-MS:  $MH^+$  638.5. IR (CaF<sub>2</sub>, neat) results:  
v<sub>as</sub> CH<sub>2</sub> 2918.3; v<sub>s</sub> CH<sub>2</sub> 2850.6; v C=O 1739.0; δ<sub>as</sub> CH<sub>2</sub>  
1467.4; v<sub>as</sub> PO<sub>2</sub> 1230.8; v C-O-C 1078.8; v<sub>s</sub> PO<sub>2</sub> 1028.2. <sup>1</sup>H  
5 NMR (500 MHz, CDCl<sub>3</sub>), δ 5.18 ppm (br s, 1H, CH), 4.35  
ppm (m, 1H, CH<sub>2</sub>OP), 4.11 ppm (m, 1H, CH<sub>2</sub>OP), 4.05 ppm (b  
s, 2H, CH<sub>2</sub>OP), 3.90 ppm (br s, CH<sub>2</sub>OCO), 3.33 ppm (t, 2H,  
OCH<sub>2</sub>), 3.30 ppm (s, 3 H, OCH<sub>3</sub>), 3.12 ppm (br s, 2H,  
NCH<sub>2</sub>), 2.28 ppm (m, 4H, CH<sub>2</sub>COO), 1.55 ppm (m, 6H  
CH<sub>2</sub>CH<sub>2</sub>COO, CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>), 1.25 ppm (br s, 34 H, (CH<sub>2</sub>)<sub>7</sub>,  
10 (CH<sub>2</sub>)<sub>10</sub>), 0.85 ppm (t, 3H, CH<sub>3</sub>).

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1 Preparation of DAC2, LPE1 and LPE2

The preparation of D-AC2 was identical to L-AC2 except D-glycerolphosphocholine, which was obtained from Synthetische Phosphor-Lipide, Biochemisches Labor, Bern CH Switzerland was used. L-PE2 was prepared by reacting glycerolphosphatidylethanolamine (L-a-GPE) with N-(9-Fluorenylmethoxycarbonyloxy) succinimide (FMOC-succinimide) to form L-a-GPE-FMOC followed by diacylation with 12MO anhydride then deprotection of FMOC with piperidine. L-PE1 was prepared by diacylating L-a-GPE-FMOC with myristic anhydride, then PLA2 cleavage to remove the sn-2 chain, then acylation with 12MO anhydride, and finally deprotection with piperidine to remove FMOC.

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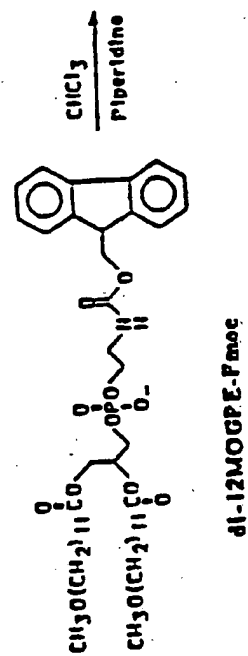
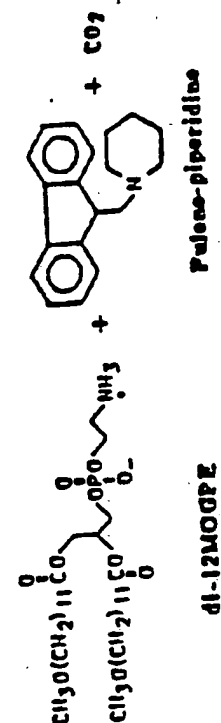
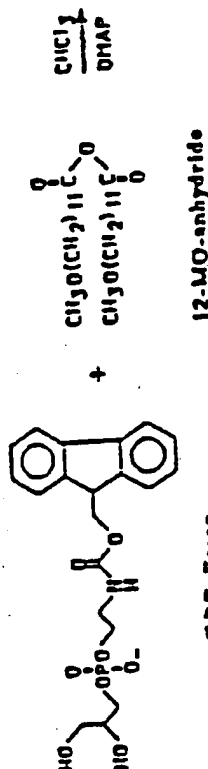
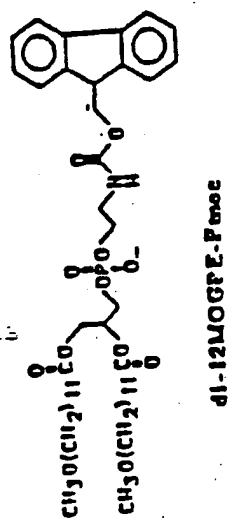
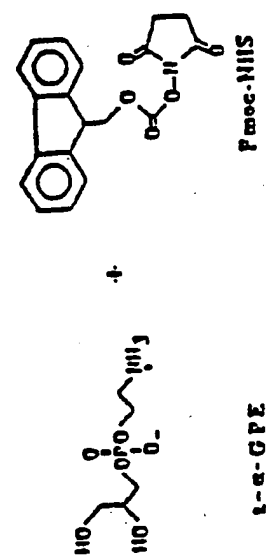
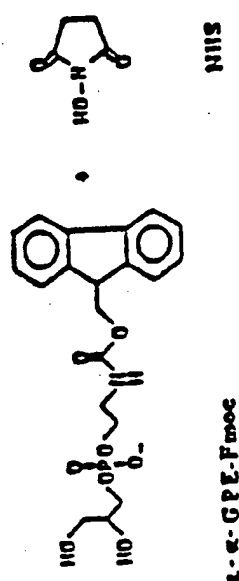
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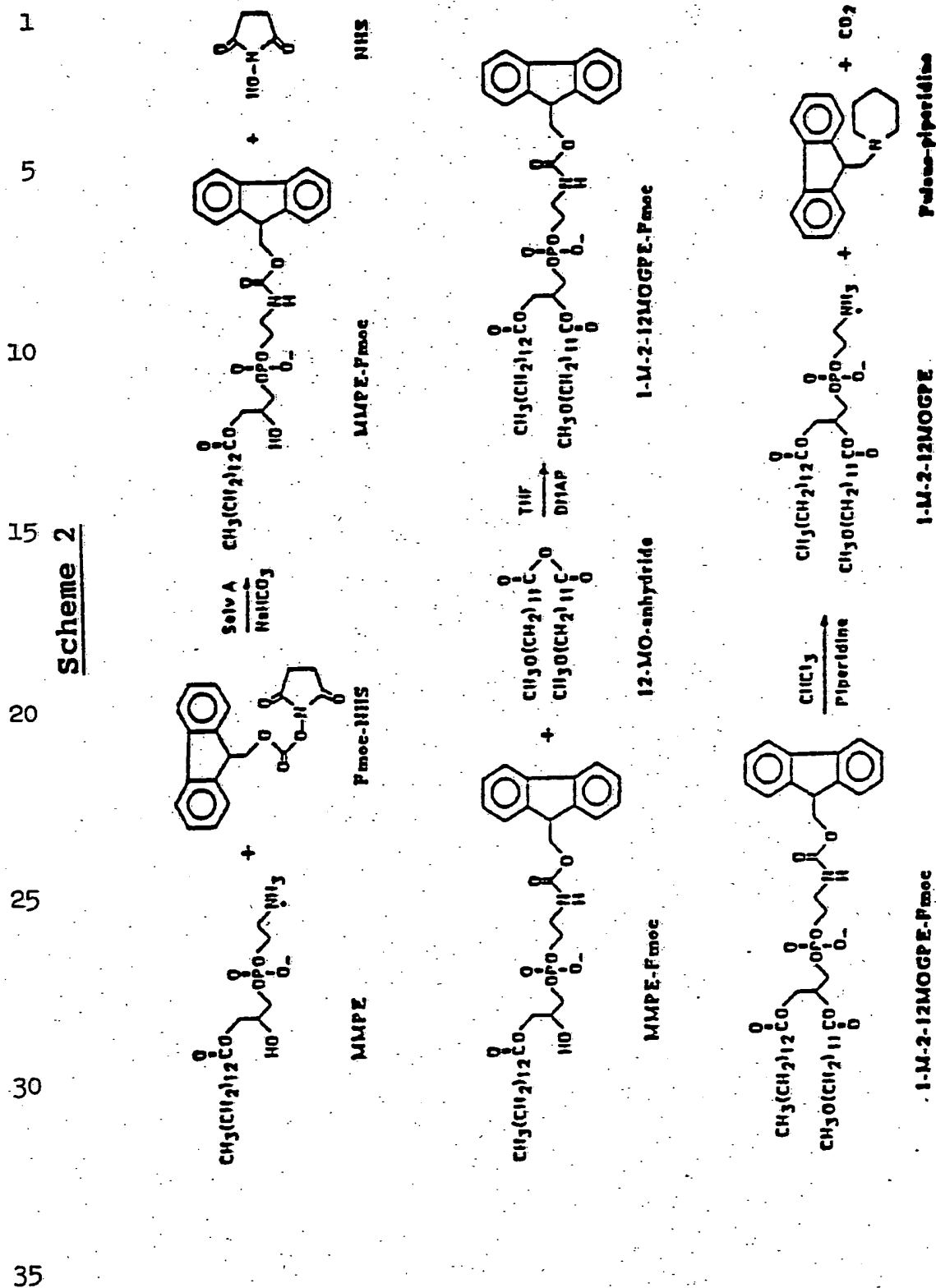
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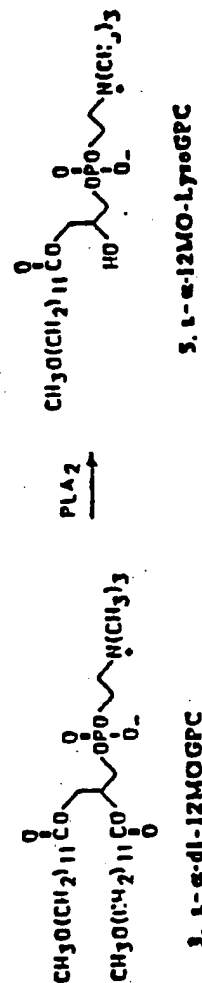
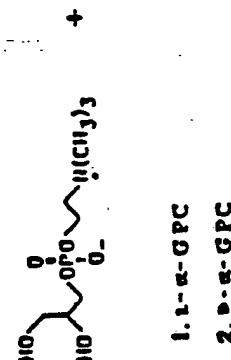
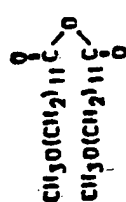
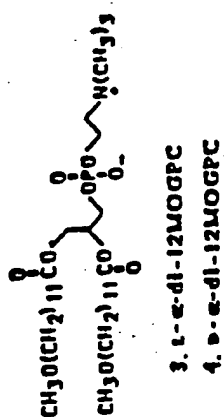
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**Scheme 1**





### Scheme 3



1           Single chain phospholipid analogs are known to  
form micelles, whereas double chain analogs form  
liposomes. After injection into animals or man,  
liposomes concentrate in macrophages, yet micelles do  
5 not. Thus, phospholipid analogs containing biologically  
active fatty acid molecules in the alkyl chains can be  
modified such that the dispersion properties of the  
phospholipids can be used to control, in part, the in  
vivo disposition of these anti-HIV drugs. Macrophages  
10 and T-cells are both CD4 positive cells and  
consequently, HIV avidly infects both of these cells.  
Thus, liposome forming anti-HIV drugs have unique  
application for combination therapy against HIV,  
particularly during viremia. If inhibitors of the HIV-  
15 CD4 binding interaction are found and employed for HIV  
therapy, then during viremia, HIV may not concentrate in  
T-Cells. However, this may cause the HIV infection to  
shift to other cells such as blood monocytes and  
macrophages, which internalize foreign particles  
20 regardless of the presence of CD4. The ability to  
control the in vivo disposition of, for e.g.,  
phosphatidylcholine anti-HIV compounds, by changing the  
number of acyl chains on the phospholipids, is therefore  
significant.

25           It is believed that liposome forming anti-HIV  
active phospholipids will most likely not be able to  
treat HIV infected tissue macrophages because liposomes  
cannot efficiently exit the blood compartment of the  
host. However, single chain lysolipid analogs which  
30 form micelles are expected to distribute into tissues  
outside of the blood compartment since lysolipid analogs



- 1 bind to albumin and are transported by albumin to  
different tissues.

The incorporation of biologically active fatty acids themselves into liposomes, for delivery to  
5 mononuclear phagocytic cells, will also concentrate the drug in blood monocytes/macrophages. However, no control over the incorporation of hetero-atom fatty acids into the cellular lipid pool exists for this delivery system. In contrast, feeding cells  
10 biologically active fatty acids covalently linked to particular phospholipids allows some control over the cellular disposition of the biologically active fatty acids. Thus a key concept for the delivery of biologically active fatty acids in the form of  
15 phospholipids is that the in vivo disposition of the drugs can be controlled by liposome forming analogs and the cellular disposition of biologically active fatty acids can be altered for therapeutic benefit by using specific phospholipid headgroups for the preparation of  
20 the drugs.

#### I. Experimental Designs and Methods

##### Overview of assay methods

Three assays for anti-HIV activity were used  
25 and the  $IC_{50}$  was calculated from these assays; (i) syncytial cell assays, (ii) reverse transcriptase assays, and (iii) direct cytotoxicity assays. In addition, direct drug cytotoxicity was tested 'as a control' in each assay which merely means that the drug  
30 was dosed to the cells without virus present. This control assured that only the antiviral effect of the compound is measured. However, this drug cytotoxicity

- 1 was not used to calculate the  $TC_{50}$  because the  $TC_{50}$   
required doses of drug that were higher than the  
effective concentrations. Thus the  $TC_{50}$  is the dose of  
drug that kills 50% of the cells without virus present  
5 and was measured in MTT assays using the same cells type  
to evaluate antiviral activity. The therapeutic index  
was calculated from  $TC_{50}/IC_{50}$ . The ability of anti-HIV  
phospholipids to inhibit direct cytopathic effect caused  
by HIV infection was also measured.

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Anti-HIV activity of AC2, AC1, lysoAC2 and 12MO  
Viral stocks

- The S5G7 strain of HIV was used. S5G7 is a  
subclone of HTLVIIIIB grown in H9 cells, and highly  
15 virulent regarding T cell infectivity, but less so for  
monocytes; this strain was obtained from Abbott  
Laboratories.

Anti-HIV activity: (1) direct cytotoxicity assay

- 20 This assay measures the ability of a drug to  
inhibit the HIV virus from killing cells. CEM cells  
were used for this assay. The protocol is given in the  
next two paragraphs.

- The T-cell HIV inhibition assay method of  
25 acutely infected cells is an automated tetrazolium based  
colorimetric assay adapted from Novak, et al., Aids Res.  
and Human Retroviruses, 6, 973 (1990). Assays were  
performed in 96-well tissue culture plates. CEM cells  
were grown in RPMI-1640 medium (Gibco) supplemented with  
30 10% fetal calf serum and were then treated with  
polybrene (2  $\mu$ g/ml). A 80  $\mu$ l volume of medium  
containing  $1 \times 10^4$  cells was dispensed into each well of

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1 the tissue culture plate. To each well was added a 100  
2  $\mu$ l volume of test compound dissolved in tissue culture  
3 medium (or medium without test compound as a control) to  
4 achieve the desired final concentration and the cells  
5 were incubated at 37°C for 1 hour. A frozen culture of  
6 HIV-1 was diluted in culture medium to a concentration  
7 of  $5 \times 10^6$  TCID<sub>50</sub> per ml (TCID<sub>50</sub> = the dose of virus  
8 that infects 50% of cells in tissue culture), and a 20  
9  $\mu$ l volume of the virus sample (containing 1000 TCID<sub>50</sub> of  
10 virus) was added to wells containing test compound and  
11 to wells containing only medium (infected control  
12 cells). This results in a multiplicity of infection of  
13 0.1 (MOI = # of infectious virus particles/# of cells in  
14 culture). Several wells received culture medium without  
15 virus (uninfected control cells). Azidothymidine (AZT)  
16 was tested as a positive drug control. Test compounds  
17 were dissolved in DMSO and diluted into tissue culture  
18 medium so that the final DMSO concentration did not  
19 exceed 1.5%. DMSO had no significant effect on results  
20 as determined in controls.

Following the addition of virus, cells were  
incubated at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere for  
7 days. Additional aliquots of test compounds were  
added on days 2 and 5. On day 7 post-infection, the  
25 cells in each well were resuspended and a 100  $\mu$ l sample  
of each cell suspension was removed for assay. A 20  $\mu$ l  
volume of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-  
2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to  
each 100  $\mu$ l cell suspension, and the cells were  
30 incubated for 4 hours at 37°C in 5% CO<sub>2</sub> environment.  
During this incubation, MTT is metabolically reduced by  
living cells resulting in the production in the cell of

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- 1 colored formazan product. To each sample was added 10  
ml of 10% sodium dodecylsulfate in 0.01N HCl to lyse the  
cells, and samples were incubated overnight. The  
absorbance of each sample was determined at 590 nm using  
5 a Molecular Devices microplate reader. The % reduction  
of the virus induced cytopathic effect (CPE) by the test  
compounds was calculated from the equation.

10  
% reduction CPE = 
$$\frac{(\text{Abs compound-treated infected sample}) - (\text{Abs of virus control})}{(\text{Abs of cell control}) - (\text{Abs of virus control})} \times 100$$

The direct cytotoxicity of each compound to  
CEM cells is labeled above each histogram bar in Figure  
1. Starting from the top graph in Figure 1, AC1 was not  
toxic to the CEM cells at dose up to 400  $\mu$ M, AC2 was  
15 toxic to CEM cells at 400  $\mu$ M, lysoAC2 was not toxic up  
to 400  $\mu$ M, and 12MO was toxic to CEM cells at 400  $\mu$ M but  
12MO also showed toxicity at 40  $\mu$ M. 12MO was the most  
toxic analog tested in this series.

20 The  $IC_{50}$  of AC2 could not be accurately  
determined from the data because the activity was too  
high but the  $IC_{50}$  is less than 4  $\mu$ Molar, and it is  
estimated that the  $IC_{50}$  is  $\sim 1$   $\mu$ M. It is also  
interesting that anchoring the biologically active fatty  
acid in the sn-2 position significantly reduced the  
25 activity (i.e., AC1 top graph), but the lysolecithin  
analog containing the biologically active fatty acid in  
the glycerol sn-1 position was active with an  $IC_{50}$   $\sim 100$   
 $\mu$ Molar. This may be due to the metabolism of lysolipids  
compared to diacylated lipids or it may indicate the  
30 myristoyl group is stored in the sn-1 chain of  
endogenous membrane lipids. AC1 and the lyso compound

- 1 were significantly less toxic than the biologically active fatty acid of AC2.

Anti-HIV activity: (2) macrophages

- 5 Anti-HIV activity was next measured in macrophages using a p24 antigen capture assay. The assay protocol is outlined below.

- Absorbance values (492 nm) for HIV-1 p24 antigen were detected by enzyme-immunoassay (EIA) in  
10 culture supernatants of HIV-1 (3B) infected monocyte-derived macrophages (MDM). Human peripheral blood MDM were prepared by adherence to plastic in 24-well tissue culture plates (Costar, Cambridge, MA). Briefly,  $1 \times 10^7$  Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient  
15 purified mononuclear cells in RPMI-1640 with 20% heat-inactivated fetal bovine serum, L-glutamine, and gentamycin (Gibco, Grand Island, NY) were placed in each well and allowed to adhere at 37°C for 3 hours. Non-adherent cells were removed by gentle washing with warm  
20 (37°C) Hank's balanced saline solution (HBSS-Gibco) and the cells incubated in a 5% CO<sub>2</sub> in air atmosphere at 37°C in 2 ml of media. Remaining non-adherent cells were further removed by washing again after 24 hours and 5 days. After 7 days in culture, the cells were  
25 infected with HIV-1 3B by removing the media from each well, washing with HBSS and adding 0.2 ml of virus-containing supernatant from a 5 day culture of a MDM permissive subclone of HIV-1 3B grown in H9 cells. The plate was rocked at 37°C for 3.5 hr, the viral inoculum  
30 removed and the cells washed 3 times each with 2 ml of warm HBSS to remove non-MDM-associated viral particles. Media containing 0, 1, 10, 50 or 100  $\mu$ M of AC1, AC2 or

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1 12MO were added to the appropriate wells and the cells  
incubated as previously described. On days 1, 3, 6, 8,  
10 and 15 after infection, 200  $\mu$ l of supernatants were  
removed from each well for p24 antigen EIA assay (HIVAG-  
5 1; Abbott Laboratories, North Chicago, IL). On day 8,  
following the sampling for p24 antigen, 1 ml of fresh  
media containing AC1, AC2, or MO was added to the  
appropriate wells to restore a concentration of 1, 10,  
50 or 100  $\mu$ M of each compound. Data is presented as  
10 absorbance values reflecting HIV-1 p24 antigen  
concentration produced by infected MDM as detected in  
culture supernatants. The experiment shown in Figure 2  
is representative of 3 experiments using 3 cell donors.  
In Figure 2 the dose of drug is given above each bar in  
15 the histogram, and -C is the negative control (no virus,  
no drug).

Figure 2 shows that AC2 was very potent and  
completely inhibited the AIDS infection in macrophages  
at all doses tested. Even at the low dose of AC2, i.e.,  
20 1  $\mu$ M, AC2 was completely effective. AC1 gave a dose  
response with almost complete inhibition at 100  $\mu$ M.  
12MO exhibited little activity except at 100  $\mu$ M where  
the HIV infection was completely suppressed. At this  
concentration, however, 12MO was toxic.

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### Anti-HIV activity: (3) Syncytial cell assays

Briefly, the assay was performed as follows.  
On Day 0, MT4 cells were infected with HIV. On Day 3,  
30 mix  $10^3$ - $10^4$  infected cells (resuspend cells at  $10^4$ - $10^5$   
cells/ml so that the desired number of cells is in a  
volume of 100  $\mu$ l) with  $10^5$  SupT1 cells (suspended at

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1  $10^6$ /ml, so that there are  $10^5$  cells per 100  $\mu$ l) in a 96  
well plate. Infected cells are titered to find the  
optimum number of syncytial cells to count. Cells are  
counted either manually or by flow cytometry. Syncytia  
5 begin to form at 8-10 hours, but optimum time for MT4  
cells is about 18 hours. The time intervals for other  
cell lines may vary. To assay drugs that block syncytia  
formation, 50  $\mu$ l Supt1, 50  $\mu$ l infected (MT4) cells  
(total cell number is the same), and 100  $\mu$ l of drug are  
10 present. Typical results are shown in Figure 3 which  
compares the  $IC_{50}$  of AC2 to 12MO. The  $IC_{50}$  of AC2 is  $\sim 1$   
 $\mu$ M and the  $IC_{50}$  of 12MO is  $\sim 4$   $\mu$ M.

15 Antiviral Activity in PHA-Lymphoblasts: (4) Reverse  
Transcriptase Assay

Mononuclear cells were obtained from the whole  
blood of normal donors by ficoll/Hypaque (Pharmacia)  
density gradient centrifugation. These cells are  
initially washed with buffer and then stimulated with  
20 PHA-M (Gibco) for 72 hours. The cells are then counted  
for number, and also viability using trypan blue  
exclusion, followed by infection with S5G7, a subclone  
of HTLVIIIIB or a wild type strain, at a multiplicity of  
infection (MOI) of 0.2 (i.e., 1 virus/5 cells) in a  
25 volume of 0.2 ml of culture supernatants for 2 hours at  
37°C. Control cells not challenged with virus are used  
to evaluate drug toxicity. Cells are then washed three  
times in RPMI +10% fetal calf serum (FCS) and plated in  
a 96-well tissue culture plate in RPMI + 10% FCS, 10mM  
30 L-GLN, 10mM Sodium pyruvate, IL-2, gentamicin. Cells  
are refluxed every 24 hours with complete media  
containing replacement drug and IL-2. Seven days after

35

1 infection, samples are taken for the reverse transcriptase assay. These samples are frozen at -70°C until analysis.

The procedure for measuring RT (reverse transcriptase) activity can be routinely performed by one of ordinary skill. Briefly, 50 µl of HIV culture supernatant are mixed with 50 µl of a 2X RT assay buffer containing Tris, 0.1M, pH 7.9, KCl, 0.32M, dithiothreitol, 0.012M, MgCl<sub>2</sub>, 0.012M, reduced glutathione, 1.2 mM, ethylene glycol-bis(beta-aminoethylether)-N,N,N',N'-tetraacetic acid, 1mM, ethylene glycol, 4%, sterile, distilled water, 10 µl, Triton X-100, 0.2%, template primer poly(rA).p(dT), 1 µ/ml, 0.05 µ/sample, (Pharmacia), and 10 µCi<sup>3</sup>H-dTTP (Amersham). Samples are incubated for 24 hours at 37°C in microtiter plates, after which the reaction is stopped with 200 µl cold 10% tetrachloroacetic acid containing 0.2 M sodium PPi. The plate is then allowed to incubate for 2 hours on ice, after which, samples are harvested onto DE-81 filter paper discs (Whatman) using a cell harvester. The discs are washed 8 times in 5% trichloroacetic acid and absolute ethanol, dried and placed into glass scintillation vials. They are then counted on a beta scintillation counter. Negative (uninfected cell supernatants are used to determine the background DNA polymerase activity, if any) and known positive controls are assayed simultaneously. Results measured in counts per minute (cpm) are plotted as % of the control (i.e., cpm obtained without drug present but with virus infection) as shown in Figure 4.



1                   Figure 4 shows that the IC50 of AC2 is -4  $\mu$ M  
and the IC50 of 12MO is -12  $\mu$ M in this reverse  
transcriptase assay.

5   Drug cytotoxicity using MTT assay (calculation of TC<sub>50</sub>)

MT4 cells were plated in 96-well microtiter  
plates at  $8 \times 10^4$  cells/well in a volume of 90  $\mu$ l.  
[Note: To compare TC50 to IC50, the same cell type used  
in antiviral assays was tested in the MTT assay; for  
10 these experiments, MT4 cells were used to evaluate the  
syncytial cell assays and PBMC's were used to compare  
the PBMC/RT assay]. To this was added one minimum  
cytotoxic dose of HIV in a volume of 10  $\mu$ l. The test  
15 drug was added in 100  $\mu$ l aliquots at several concentra-  
tions. A control plate using uninfected MT4 cells was  
set up simultaneously to assess cytotoxicity due to the  
drug alone. The plates were then incubated for five  
days at 37°C. Then the media was aspirated from the  
wells and replaced with 100  $\mu$ l MTT solution (3-(4,5-  
20 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,  
1mg/ml in PBS) and incubated for 4 hours at 37°C. The  
plates were then centrifuged to pellet the cells, and  
the supernatants were removed (centrifugation can be  
omitted if the supernatants are removed carefully).  
25 Acidic isopropanol (0.04N HCl in isopropanol), 100  $\mu$ l,  
was then added to the wells and shaken for 15-30 minutes  
to dissolve the formazan crystals. Plates were read on  
an ELISA reader at Abs 570 nm. The difference between  
the uninfected and infected plates represent the  
30 antiviral activity of the drug. This assay was used to  
determine drug toxicity alone. A similar method has

- 1 been described in the AIDS Research and Reference  
Reagent Program Courier, 90-01:8-9, 1990.

Figure 5 shows the results of a typical  
experiment run in MT 4 cells which can be compared  
5 directly to the syncytial cell assay shown in Figure 3  
because the same cell line was used. The TC50 of AC2  
was 280  $\mu$ M and the TC50 of 12MO was 140  $\mu$ M as shown in  
Figure 5.

10 Therapeutic Index of Lipids having anti-HIV-Activity

Table 1 summarizes the data shown in Figure 1-  
5 by comparing the therapeutic index for 12MO and AC2.  
The therapeutic index compares the toxic dose (TC50) to  
the effective dose (IC50). The therapeutic index values  
15 given in Table 1 are calculated from toxicity data and  
activity data that were obtained in the same cell line.  
In syncytial cell assays using MT 4 cells the  
therapeutic index for AC2 is 280 whereas for 12MO the  
therapeutic index is only 35. In PBMC's, the  
20 therapeutic index for AC2 is 31 whereas for 12 MO the  
therapeutic index is 37.

Moreover, L-AC2 was significantly more potent  
than 12MO in HIV infected monocyte-derived macrophages  
(MDM) shown in Figure 1. During a 15 day acute  
25 infection, L-AC2 at 1  $\mu$ M completely suppressed the HIV  
infection in MDM yet 12MO had little activity at doses  
from 1 to 50  $\mu$ M. However, the 100 $\mu$ M dose of 12MO  
suppressed HIV replication in MDM which demonstrates  
that 12MO exhibited a very steep dose response effect in  
30 MDM i.e., little activity at 50 $\mu$ M and virtually 100%  
activity at 100  $\mu$ M. No dose response was observed in

- 1 MDM for L-AC2 because the lowest dose of L-AC2 tested (1  $\mu$ M) completely inhibited the HIV infection in MDM.

In HIV infected MDM, L-AC1 exhibited dose responsive activity and 50  $\mu$ M L-AC1 completely  
5 suppressed HIV p24 antigen production; this dose of L-AC1 had little activity in CEM cells using a direct cytopathic assay (Figure 1). Diacylated phospholipids form liposomes, and as expected both L-AC1 and L-AC2  
10 formed liposomes in aqueous buffers. The increased activity of L-AC1 in MDM compared to CEM cells may be due to the phagocytosis of L-AC1 liposomes in MDM.

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Table I. IC<sub>50</sub> and Therapeutic Index of L-AC2 and 12MO

	TC <sub>50</sub>	IC <sub>50</sub> (b)	Therapeutic Index (c)	IC <sub>50</sub> (b)	Therapeutic Index (c)
	Cytotoxicity (a) MTT Assay	50% Reduction in syncytial cell formation MT4 cells (a)	MT4 cells	50% Reduction in Reverse Transcriptase Activity PBMC (a)	PBMC
	$\mu\text{M}$	$\mu\text{M}$		$\mu\text{M}$	
12 MO	140 (MT4 cells)	4	35		
	340 (PBMC cells)			19	17
L-AC2	280 (MT4 cells)	1	280		
	220 (PBMC cells)			6	37

SUBSTITUTE SHEET

- 1 a) Drug cytotoxicity in the absence of HIV was determined using MT4 cells or PBMC's. The  $TC_{50}$  is the concentration of the drug that killed 50% of the cells during a 4 hour incubation period.
- 5 b) The  $IC_{50}$  is the drug concentration that inhibited 50% of the maximum HIV response which was observed when no drug was present. The HIV responses that were measured are syncytial cell formation of reverse transcriptase activity.
- c) The therapeutic index was calculated as  $TC_{50}/IC_{50}$ .
- 10 d) Syncytial cell assays were performed using MT4 cells infected with HIV and SupT1 cells as target cells. MT4 cells are CD4+ and highly susceptible to HIV infection.
- e) Drug activity was determined in PBMC by measuring inhibition of reverse transcriptase activity.
- 15 Compounds of the present invention (IV, V, VI), especially those containing at least one heteroatom fatty acid acyl chain, exhibit synergistic effects when administered with AZT. The more preferred embodiments (compounds of formulae VIII-XV) also exhibit this
- 20 effect. An illustrative example is given hereinbelow.

#### Synergism of AC2 with AZT

Another set of data demonstrates the synergism observed with AC2 and AZT in syncytial cell assays using T cells.

12MO has been reported to act synergistically when administered concurrently with AZT, and the synergism between AZT and either 12MO or L-AC2 using syncytial cell assays was evaluated. (Figure 6). The dose response curves for each drug alone shows that AZT is ~100 times more potent than L-AC2; however, AZT is ~ $10^3$  to  $10^4$  times more potent than 12MO. Synergism was

1 evaluated by the shift in the dose response curves when  
0.5 nM AZT (an inactive concentration) was added to 12MO  
and L-AC2; 12MO exhibited less than a factor of 10  
increase in activity whereas L-AC2 exhibited  
5 approximately 100 fold increased activity (Figure 6).  
For instance, Figure 6 shows that 5 nM L-AC2 (an  
inactive concentration labelled \*\* in Figure 6) and 0.5  
nM AZT (an inactive concentration) exhibited ~50%  
inhibition of syncytia formation in HIV infected MT4  
10 cells. Figure 6 also shows that 10 nM L-AC2 (an  
inactive concentration) shifts the dose response curve  
of AZT approximately 100 fold. Using MT4 cells,  
concentrations of AZT from 50 nM to 500 nM did not alter  
the concentration of L-AC2 that killed 50% of uninfected  
15 cells, i.e., the  $TC_{50}$  of L-AC2 is unchanged in the  
presence of AZT.

Figure 6 clearly shows the increased  
synergistic effects of L-AC2 with AZT as compared with  
12MO and AZT. Without wishing to be bound, it is  
20 believed that the synergism for L-AC2 and AZT is not due  
to increased cellular toxicity from administering both  
drugs concurrently; it is believed that the synergism is  
due to direct inhibition of HIV by two different  
mechanisms. AZT is a reverse transcriptase inhibitor;  
25 whereas, L-AC2 putatively inhibits endogenous  
myristoylation of the HIV proteins.

#### Anti-HIV Activity of D-AC2

L-AC2 contains the natural configuration of  
30 glycerophosphocholine and is quantitatively hydrolyzed  
by bee venom phospholipase A2 (PLA2) within minutes.  
PLA2s stereospecifically hydrolyze phospholipids.

1 However, the D-isomer, i.e., D-AC2 was prepared to test  
the hypothesis whether endogenous PLA2s are responsible  
for 12MO release from phosphatidylcholine analogs  
containing 12MO. Figure 7 shows that the  $IC_{50}$  for D-AC2  
5 is  $\sim 1$  uMolar which is identical to the  $IC_{50}$  of L-AC2.  
Unlike L-AC<sub>2</sub>, D-AC2 is not hydrolyzed by PLA2.

Figure 7 also shows the anti-HIV activity of  
the phosphatidylethanolamine (PE) analogs L-PE1 and L-  
PE2; these analogs are chemically similar to L-AC1 and  
10 L-AC2 respectively except the PC headgroup has been  
changed to the PE headgroup. The  $IC_{50}$  of L-PE1 and L-  
PE2 are 6 uMolar and 0.02 uMolar respectively; compared  
to the PC analogs this is approximately a 20-50 fold  
increase in activity. The anti-HIV activity of L-PE2 is  
15 > 100 fold more than 12MO (Figure 7).

Stability of Anti-HIV Phospholipids in Fresh Blood  
at 38°C

Drug development using phospholipids will  
20 require that the parent compound is stable in blood.  
Figure 8 shows that the halflife of L-AC2 in fresh blood  
is 4.56 hours and the halflife of D-AC2 is 18.24 hours  
( $\sim 4$  times longer). Thus, by changing the  
stereochemistry of the glycerobackbone to the unnatural  
25 configuration the halflife in blood can significantly be  
increased. Changing the lipid headgroup also increases  
the stability in blood. L-PE2 has a halflife in fresh  
blood of 9.36 hours which is approximately 2 times  
longer than L-AC2 (Figure 8). It was very surprising  
30 that L-PE1 has a very long halflife ( $T_{1/2}$  > 50 hours)  
compared to the L-PE2. L-PE1 and L-PE2 are identical  
except that the methylene group in the 13 position of

- 1 the sn-2 alkyl chain has been replaced with an oxygen atom.

- Without wishing to be bound, it is believed that the increased activity, particularly of L-AC2 and
- 5 L-PE2 compared to 12MO, and also the increased synergism of L-AC2 with AZT compared to 12MO and AZT is due to the cellular disposition of phospholipid analogs. When 12MO is delivered to cells as a free fatty acid it is rapidly incorporated into triglycerides and membrane lipids.
- 10 The  $T_{1/2}$  for incorporation is approximately 1-2 minutes. Triglycerides are usually thought of as storage depot for fats that are used as a source of energy. If intracellular triglycerides containing 12MO are used primarily as an energy source instead of a source of
- 15 fatty acids for myristoylation of HIV proteins, then this may be the primary reason why the cellular availability, necessary for anti-HIV activity, of 12MO is 10 fold or 100 fold less than L-AC2 and L-PE2. Thus lipid metabolism and the intracellular disposition of
- 20 hetero atom fatty acids and anti-HIV phospholipids can significantly affect anti-HIV activity.

- The data in the figures and the Table clearly illustrate that acylation of a drug containing a carboxy group to the hydroxy group of the glycerol backbone of a
- 25 phospholipid significantly enhances the pharmacokinetics of said drug. The phospholipid drug has an increased therapeutic index relative to the non-phospholipid drug. The phospholipid drugs prepared in accordance with the present invention can be more potent (Figure 1), less
- 30 toxic (Figure 1), and more stable (Figure 8), and can have increased availability or distribution relative to the non-phospholipid drug. The enhanced pharmacokinetics



1 of the phospholipid drugs prepared in accordance with  
the present invention makes it an extremely powerful  
weapon in the war against diseases.

The foregoing description of the invention has  
5 been presented for purposes of illustration and  
description and is not intended to be exhaustive or to  
limit the invention to the precise form disclosed.  
Other variations are possible in light of the teachings  
presented herein.

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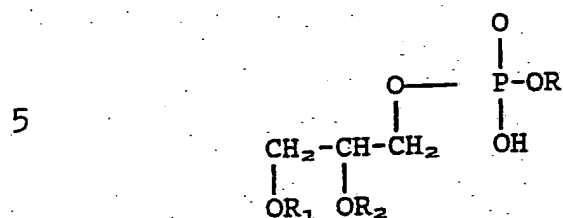
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1 WHAT IS CLAIMED IS:

1. A compound of the formula



or pharmaceutically acceptable salts thereof

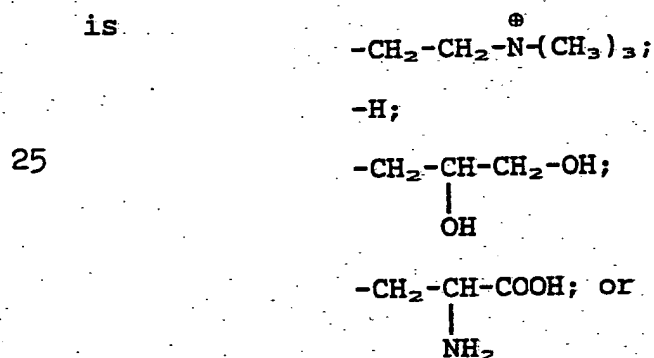
wherein

10 one of  $R_1$  and  $R_2$  is a heteroatom fatty acid acyl group having 13-14 carbon atoms in the principal chain and up to a total of 18 carbon atoms, while the other is hydrogen, a heteroatom of fatty acid acyl group containing 13-14 carbon atoms in the principal chain and

15 up to a total of 18 carbon atoms or an acyl group of a fatty acid containing 4-26 carbon atoms in the principal chain and up to a total of 30 carbon atoms and

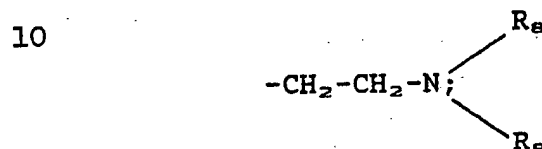
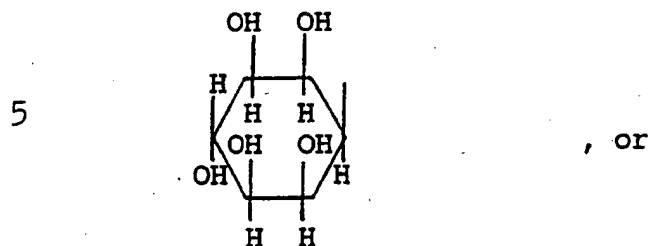
20  $R$  is a naturally occurring polar group characteristic of a glycerolphospholipid isolated from endogenous sources.

2. The compound according to Claim wherein  $R$  is



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1                                     $-\text{CH}_2-\text{CH}_2-\text{NH}_2$ , or



15                                    3. The compound according to any one of Claims 1-2 wherein the acyl group of a fatty acid contains 4-16 carbon atoms.

20                                    4. The compound according to any one of Claims 1-3 wherein  $\text{R}_1$  is a heteroatom fatty acid acyl group and  $\text{R}_2$  is hydrogen, a heteroatom fatty acid acyl group or an acyl group of a fatty acid.

                                     5. The compound according to any one of Claims 1-4 wherein  $\text{R}_2$  is a heteroatom fatty acid acyl group or hydrogen.

25                                    6. The compound according to any one of Claims 1-3 wherein  $\text{R}_2$  is a heteroatom fatty acid acyl group and  $\text{R}_1$  is hydrogen, a heteroatom fatty acid acyl group or an acyl group of a fatty acid containing 4-14 carbon atoms.

30                                    7. The compound according to any one of Claims 1-3 and 6 wherein  $\text{R}_1$  is a heteroatom fatty acid acyl group, hydrogen, or an aryl group of a fatty acid containing 4-14 carbon atoms.

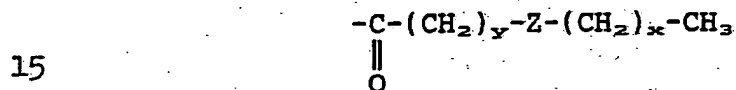
35                                    8. The compound according to any one of Claims 1-7 wherein  $\text{R}_1$  and  $\text{R}_2$  are both a heteroatom fatty acid acyl group.

1           9. The compound according to any one of  
Claims 1-8 wherein  $R_1$  is the same as  $R_2$ .

5           10. The compound according to any one of  
Claims 1-3 wherein  $R_1$  is a heteroatom fatty acid acyl  
group and  $R_2$  is hydrogen or an acyl group of a fatty  
acid containing 4-14 carbon atoms.

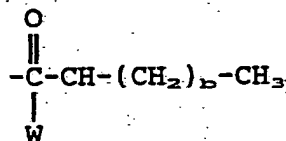
10           11. The compound according to any one of  
Claims 1-3 wherein  $R_2$  is a heteroatom fatty acid acyl  
group and  $R_1$  is hydrogen or an acyl group of a fatty  
acid containing 4-14 carbon atoms.

12. The compound according to any one of  
Claims 1-11 wherein the heteroatom fatty acid acyl group  
has the formula:



wherein  $x$  is 0-11 and  $y$  is 1-11 and  $x+y = 11$  and  $Z$  is O  
or S.

20           13. The compound according to any one of  
Claims 1-11 wherein the heteroatom fatty acid acyl group  
has the formula:



25           wherein  $W$  is hydroxy, halo, lower alkoxy, mercapto or  
lower alkylthio; and

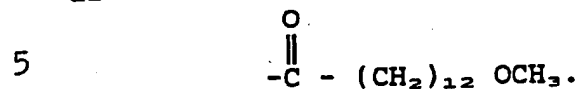
$b$  is 11.

14. The compound according to Claim 13 wherein  
 $W$  is hydroxy or halo.

30           15. The compound according to Claim 14 wherein  
halo is Br or Cl.

35

- 1            16. The compound according to any one of  
 Claims 1-12 wherein the heteroatom fatty acid acyl group  
 is



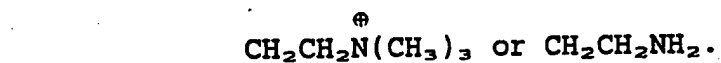
17. The compound according to any one of  
 Claims 1-16 wherein the acyl group of the fatty acid is  

$$\begin{array}{c} \text{C}(\text{CH}_2)_{12}\text{CH}_3. \\ \parallel \\ \text{O} \end{array}$$
- 10

18. The compound according to any one of  
 Claims 1-17 wherein R is  

$$\begin{array}{c} \text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+, \text{CH}_2\text{CH}_2\text{NH}_2, \text{H}, \\ \text{CH}_2\text{CH}-\text{CH}_2\text{OH}, \text{ or } \text{CH}_2-\text{C}^+-\text{COOH} \\ | \qquad \qquad \qquad | \\ \text{OH} \qquad \qquad \qquad \text{NH}_2 \end{array}$$
- 15

19. The compound according to any one of  
 Claims 1-18 wherein R is



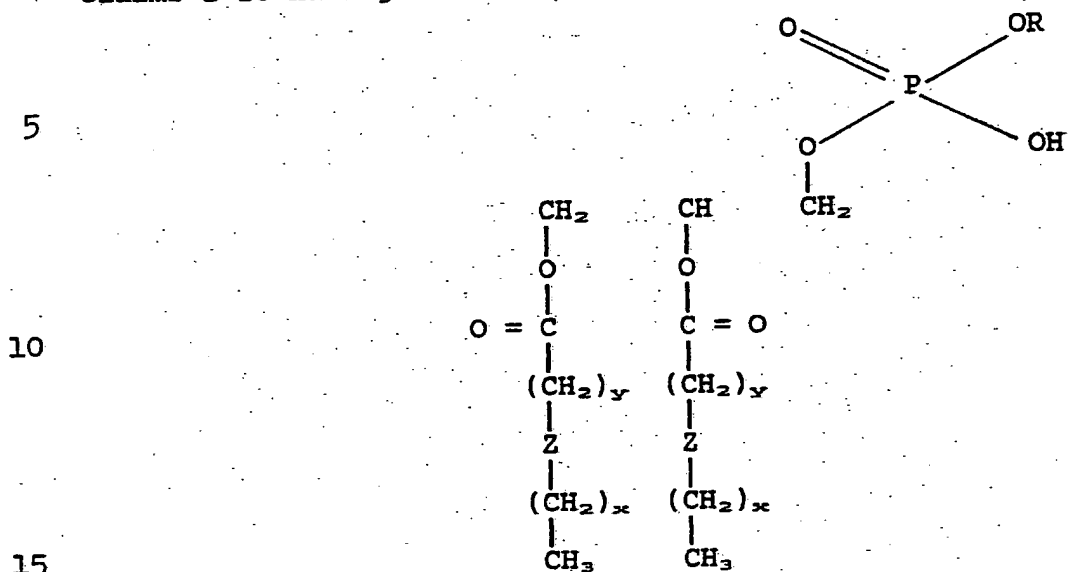
20. The compound according to any one of  
 Claims 1-19 wherein Z is O.

25

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- 1            21. The compound according to any one of  
 Claims 1-20 having the formula:



or pharmaceutically acceptable salts thereof  
 wherein

- 20            R is a naturally occurring polar head group  
 characteristic of a glycerophospholipid isolated from  
 endogenous sources;

- R<sub>1</sub> and R<sub>2</sub> are independently hydrogen or an  
 alkyl fatty acid acyl group having 4-26 carbon atoms,  
 and A is a heteroatom alkyl fatty acid acyl group having  
 25    3-25 carbon atoms

            Z is O or S;

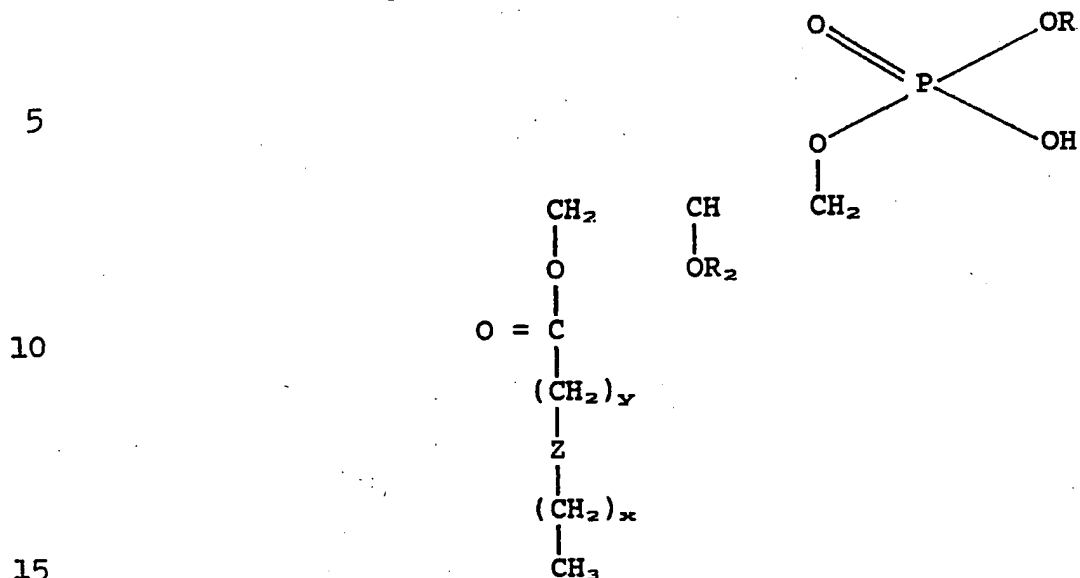
            x = 0-11

            y = 1-11 and x + y = 11.

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- 1            22. The compound according to any one of  
 Claims 1-20 having the formulae:



or pharmaceutically acceptable salts thereof  
 wherein

R is a naturally occurring polar head group  
 characteristic of a glycerophospholipid isolated from  
 20 endogenous sources;

Z is O or S;

x = 0-11,

y = 1-11, and x + y = 11, and

25 R<sub>1</sub> and R<sub>2</sub> are independently hydrogen or  $\begin{array}{c} \text{C} \\ \parallel \\ \text{O} \end{array} - \text{R}_7$ ;

and

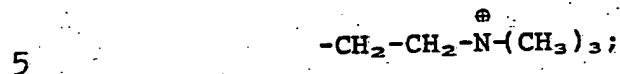
R<sub>7</sub> is an alkyl group containing 3-18 carbon  
 atoms.

- 30            23. The compound according to Claim 22 wherein  
 R<sub>7</sub> is an alkyl group containing 13 carbon atoms.

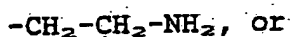
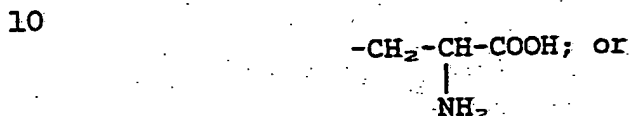
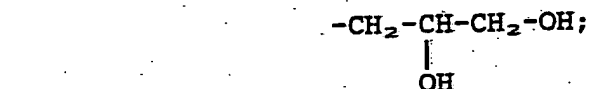
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- 1            24. The compound according to any one of  
Claims 21-23 wherein

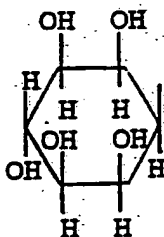
R is



$-\text{H};$

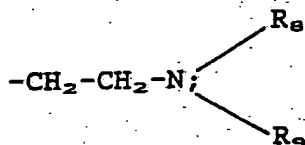


15



, or

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25. The compound according to any one of  
Claims 21-24 wherein R is  $\text{CH}_2\text{CH}_2\overset{\oplus}{\text{N}}(\text{CH}_3)_3$  or  $\text{CH}_2\text{CH}_2\text{NH}_2$ .

- 30           26. The compound according to any one of  
Claims 21-25 wherein Z is O.

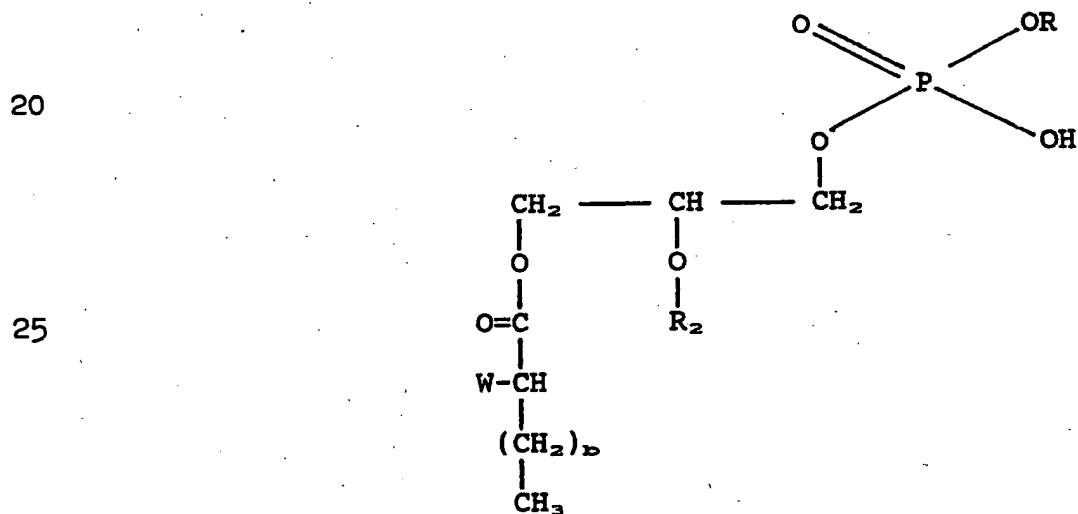
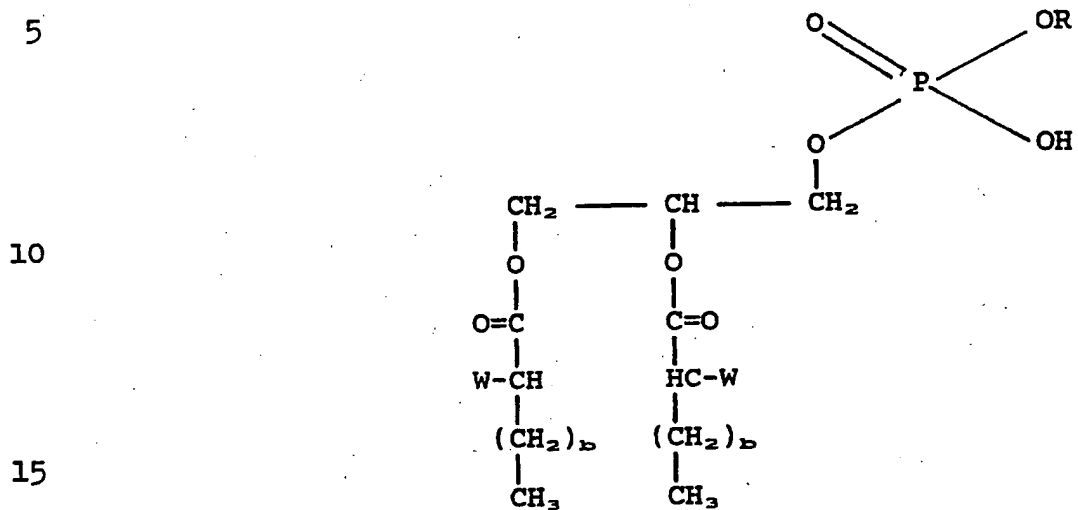
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1 27. The compound according to any one of  
Claims 21-26 wherein x is 0 and y is 11.

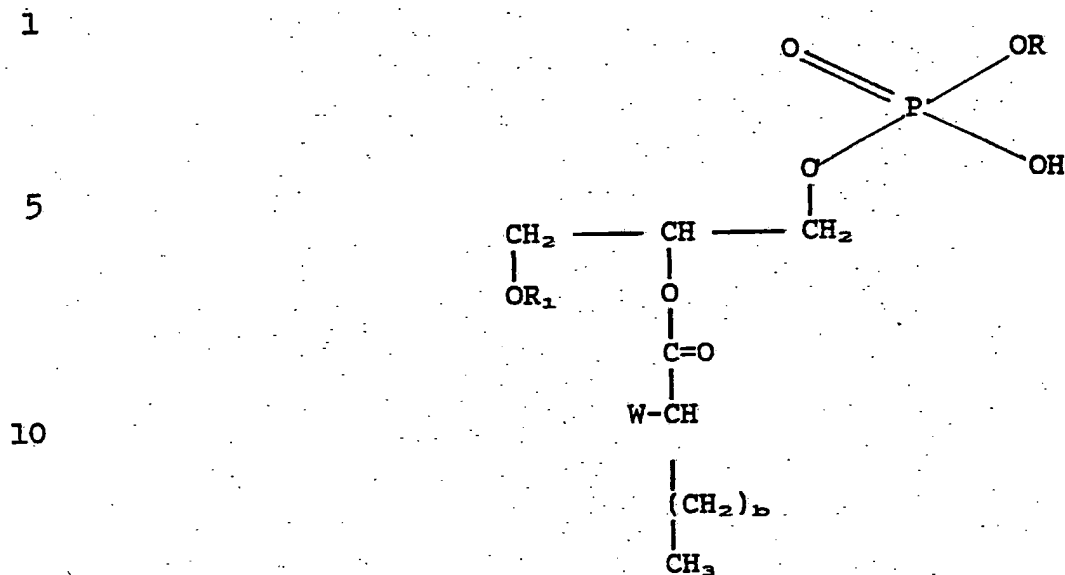
28. The compound according to any one of  
Claims 1-9 having the formulae:



or

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## XII

or pharmaceutically acceptable salts thereof  
wherein

W is hydroxy, halo, lower alkoxy, mercapto or  
lower alkylthio,

20

b is 11

R is a naturally occurring polar head group  
characteristic of a glycerophospholipid isolated from  
endogenous sources;

25

R<sub>1</sub> and R<sub>2</sub> are independently hydrogen or  $\text{C} - \text{R}_7$ ,  
 $\begin{array}{c} \parallel \\ \text{O} \end{array}$

R<sub>7</sub> is an alkyl group containing 3-18 carbon  
atoms.

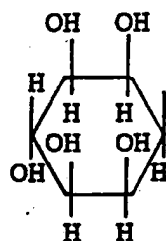
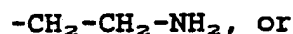
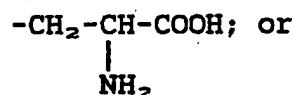
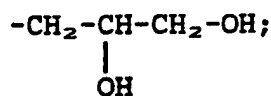
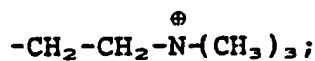
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29. The compound according to Claim 28  
wherein W is hydroxy or halo.

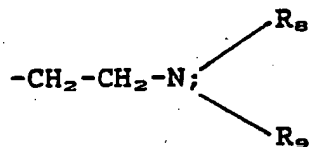
30. The compound according to Claim 29  
wherein halo is Br or Cl.

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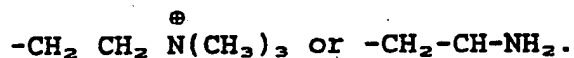
1            31. The compound according to any one of  
Claims 28-30 wherein R is



, or

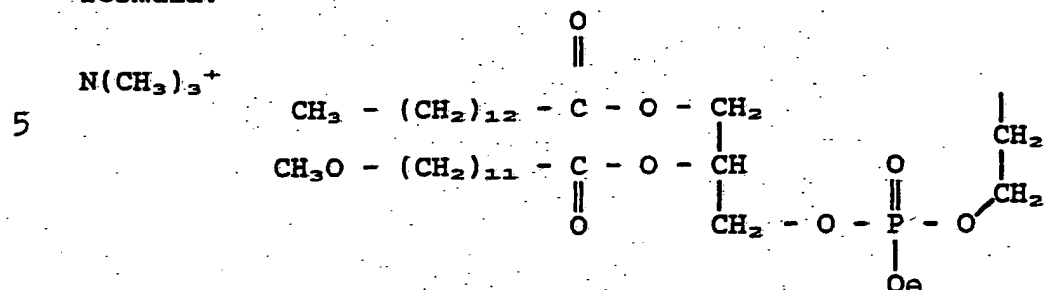


25                    32. The compound according to any one of  
Claims 28-31 wherein R is

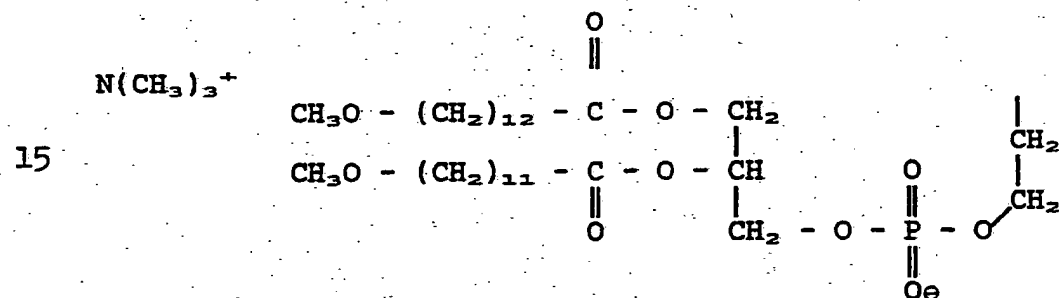


30 33. The compound according to any one of  
Claims 28-32 wherein R<sub>7</sub> is an alkyl group containing 3-7  
or 13 carbon atoms.

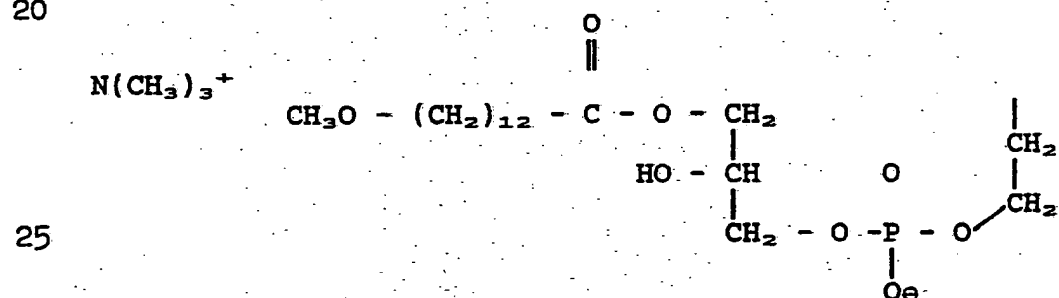
- 1 34. The compound according to Claim 1 of the  
formula:



- 10 35. The compound according to Claim 1 of the  
formula:



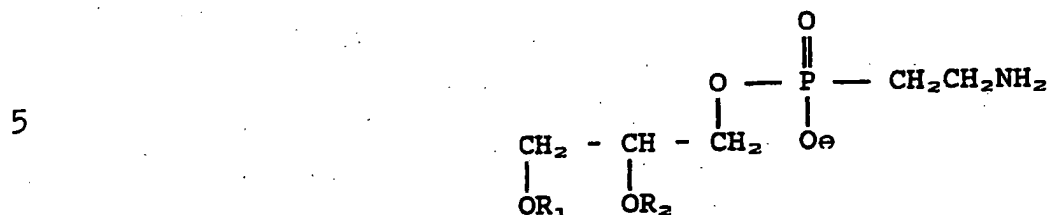
- 20 36. The compound of Claim 1 of the formula:



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1                    37. The compound according to Claim 1 which  
                      is



wherein

10                     $R_1$  is myristoyl or 12-methoxydodecanoyl and  
                       $R_2$  is 12-methoxydodecanoyl acid.

38. The compound according to any one of  
 Claims 1-37 wherein the configuration at the carbon in  
 the Sn-2 position is L.

15                    39. The compound according to any one of  
 Claims 1-37 wherein the configuration at the carbon in  
 the Sn-2 position is D.

40. A substantially pure compound of any of  
 Claims 1-39.

20                    41. A method for the inhibition of protein  
 myristoylation in animals comprising administering to  
 said animal an effective amount of a compound according  
 to any one of claims 1-40.

42. A method for the inhibition of retroviral  
 25 proliferation in an animal comprising administering to  
 said animal a retroviral proliferation inhibiting amount  
 of the compound according to any one of Claims 1-40.

43. A method for the treatment of AIDS in an  
 animal which comprises administering to said animal an  
 30 anti-AIDS effective amount of the compound according to  
 any of Claims 1-40.

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1           44. A pharmaceutical composition suitable for  
administration to an animal in need thereof comprising a  
pharmaceutically acceptable carrier and an effective  
amount of a compound according to any one of Claims 1-  
5   40.

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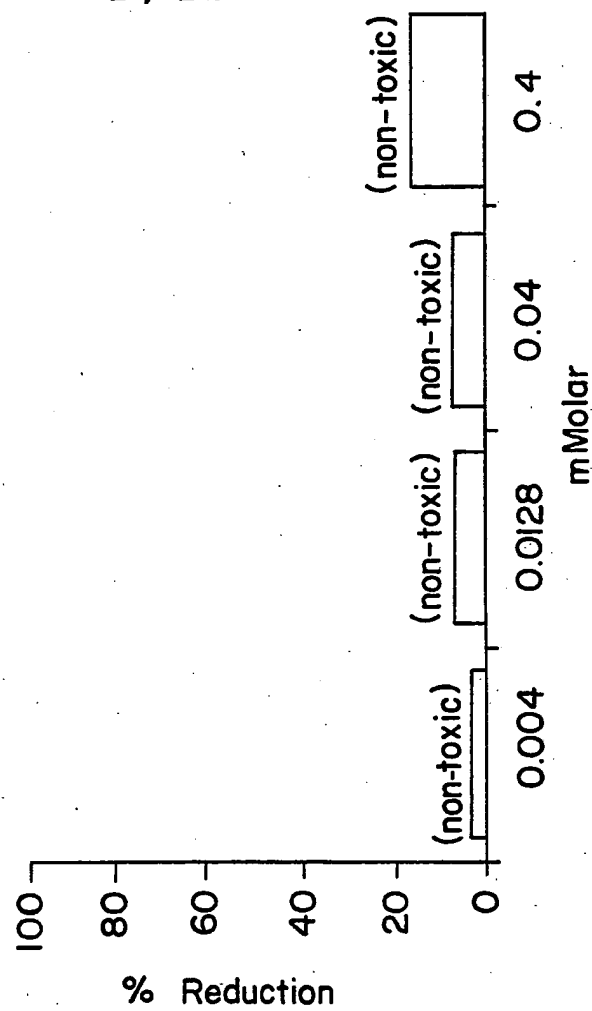
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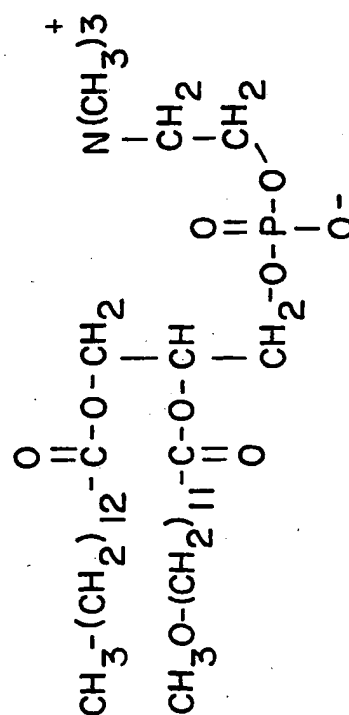
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FIG. 1a

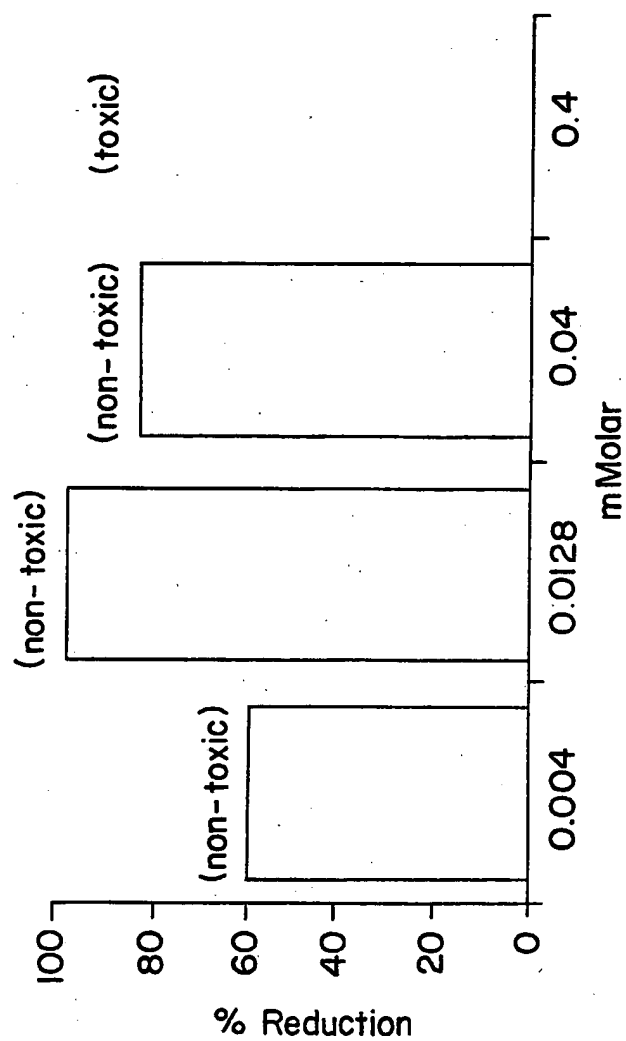


ACI



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FIG. 1b



AC2

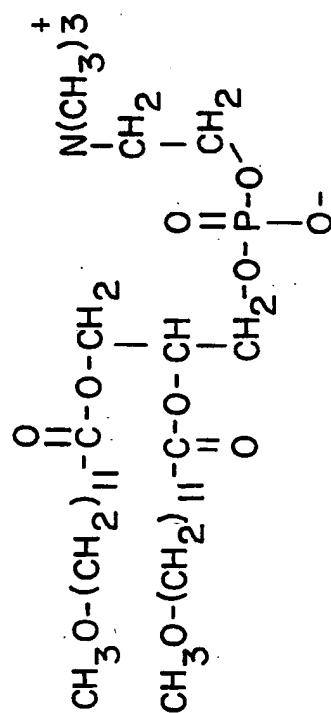
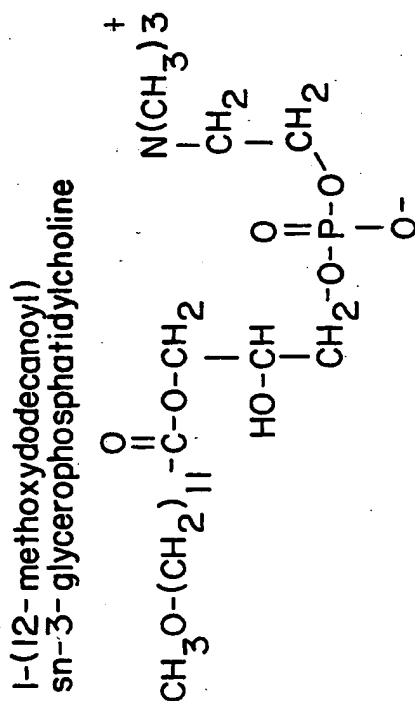
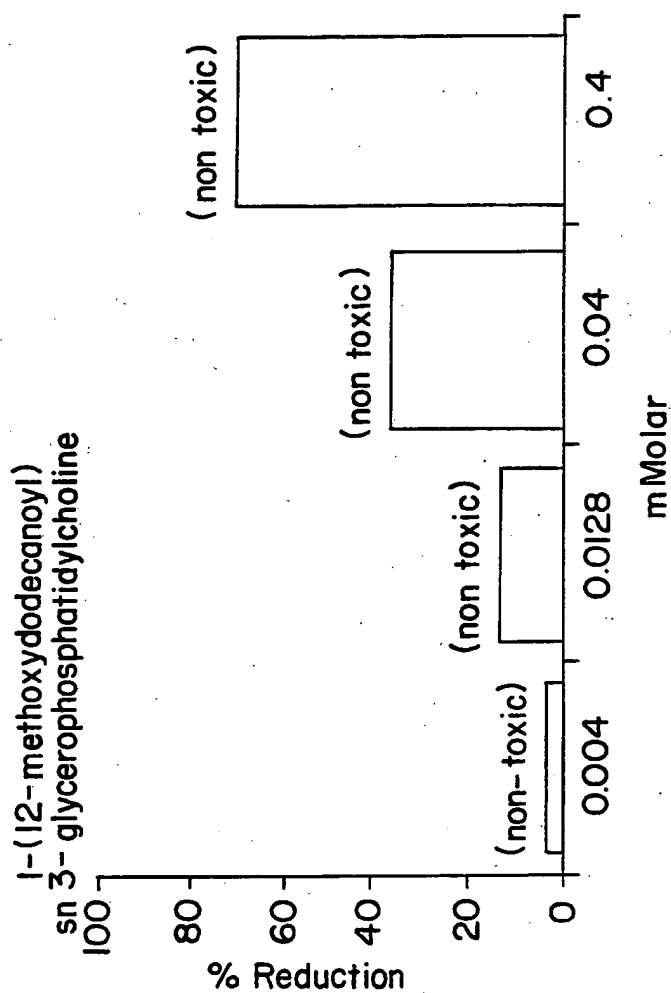


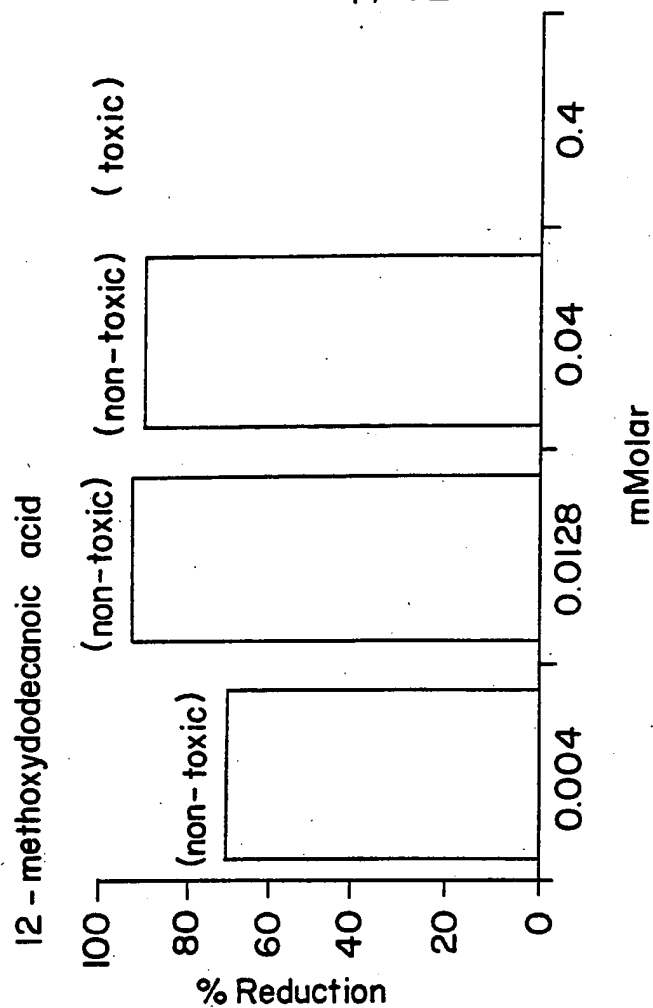


FIG. 1c



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FIG. 1d



12-methoxydodecanoic acid

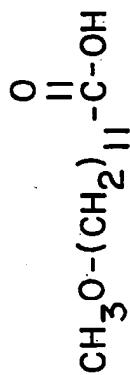


FIG. 2A

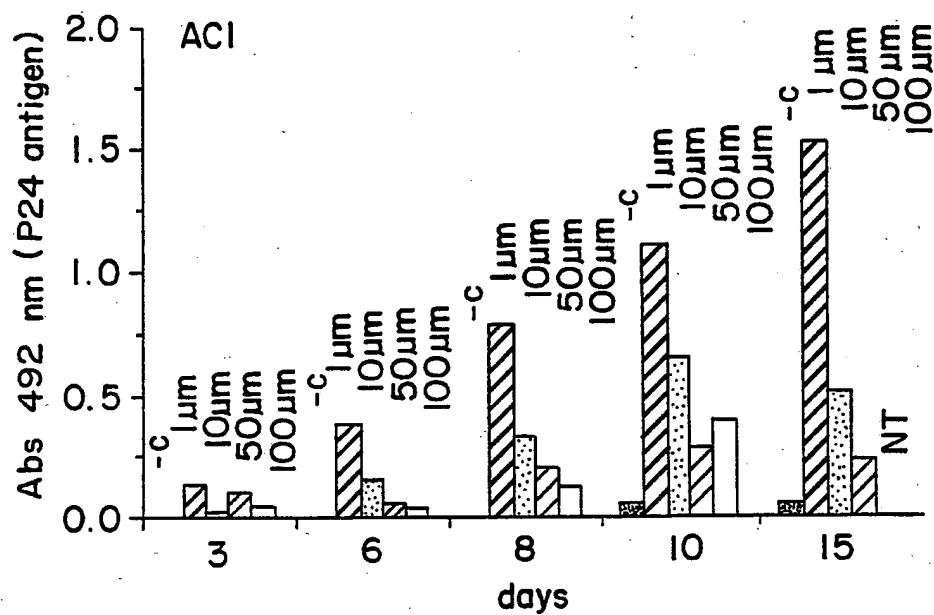
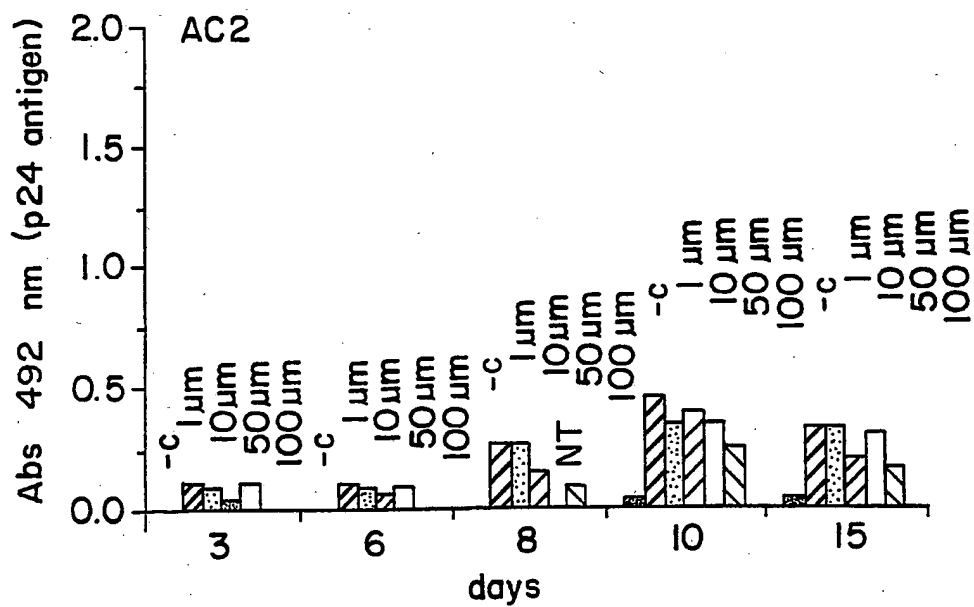


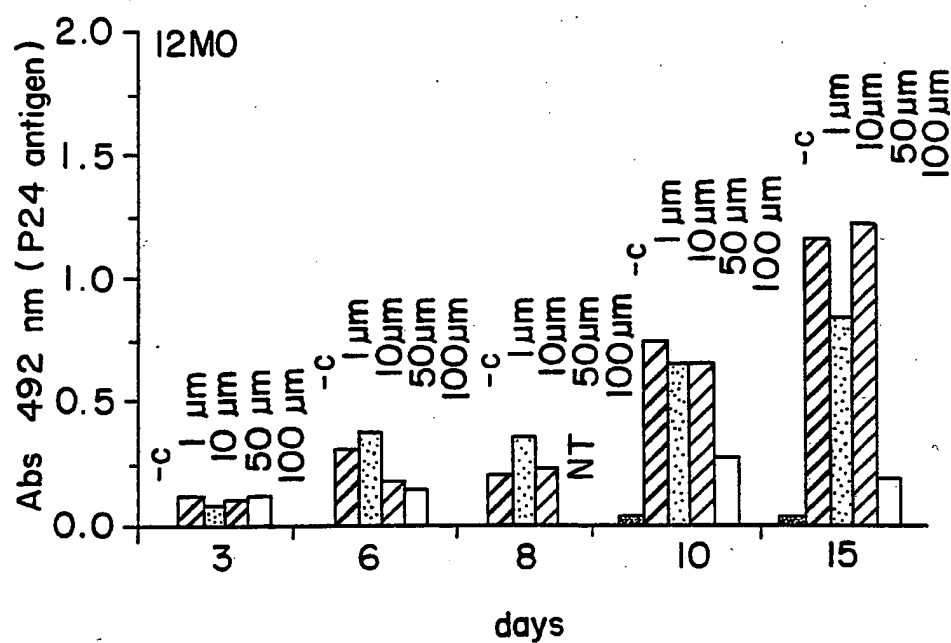
FIG. 2B



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FIG. 2C



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FIG. 3A

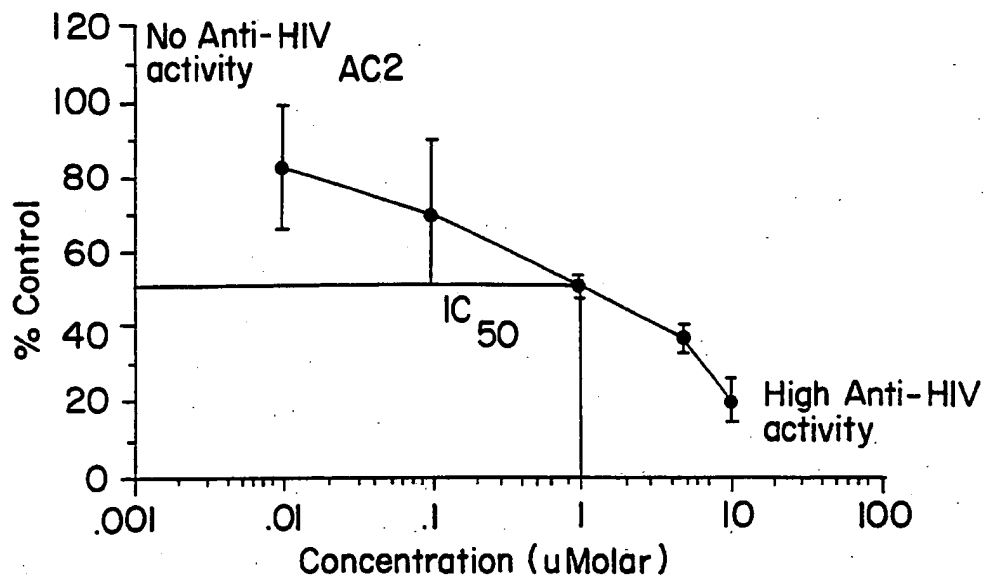
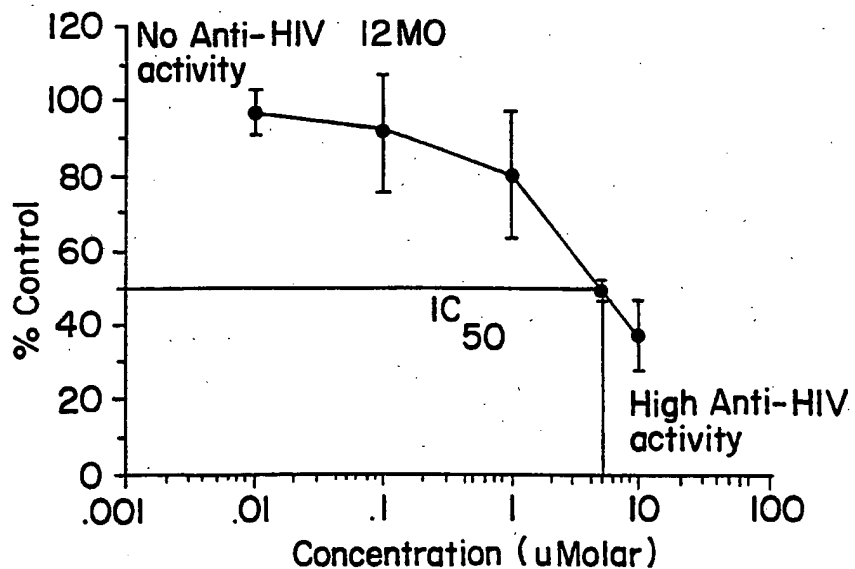


FIG. 3B



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FIG. 4A

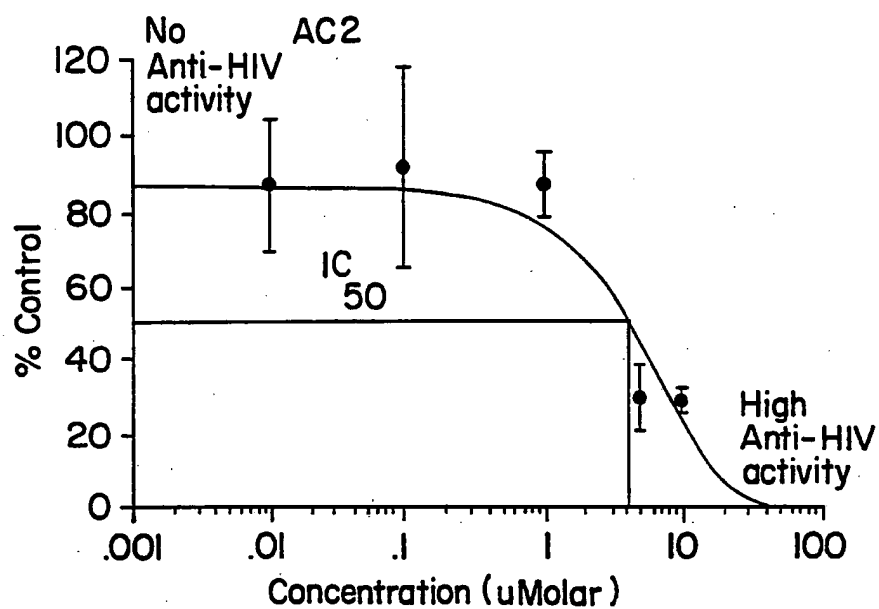
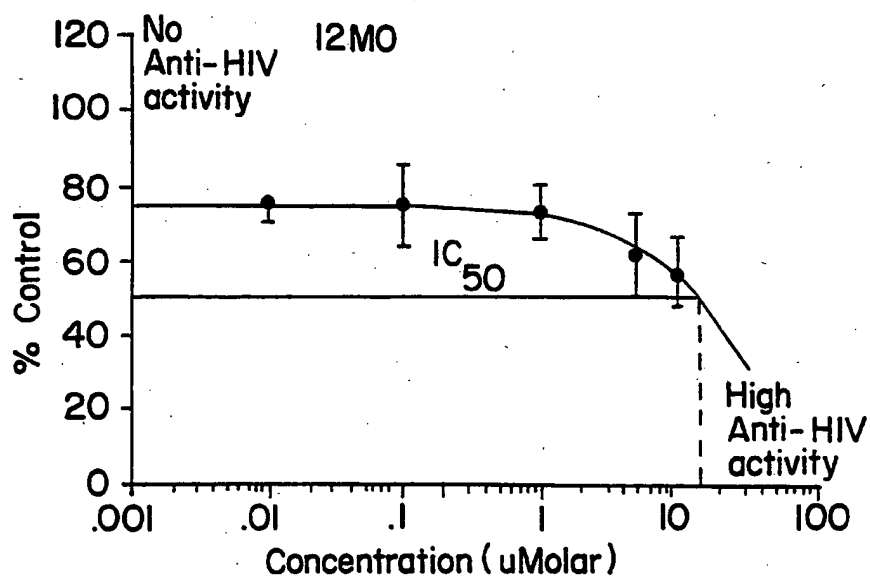


FIG. 4B



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FIG. 5A

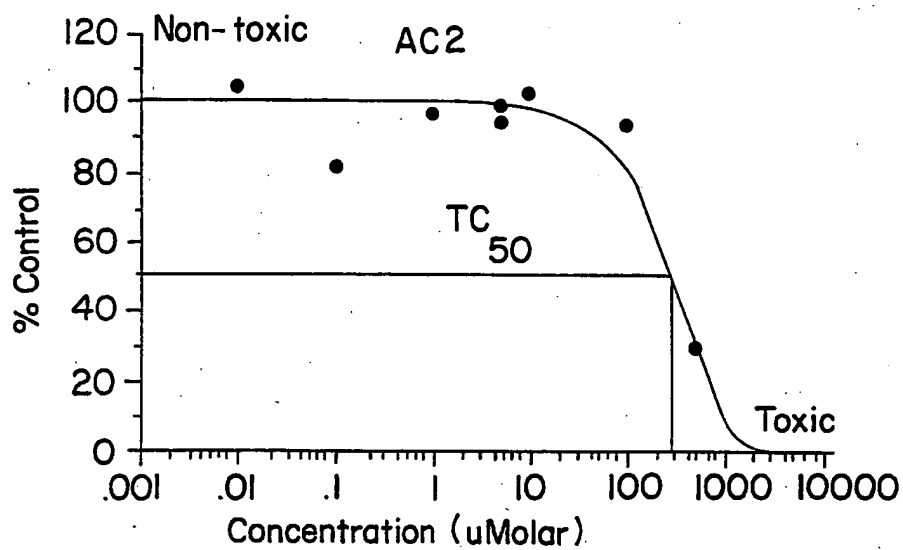
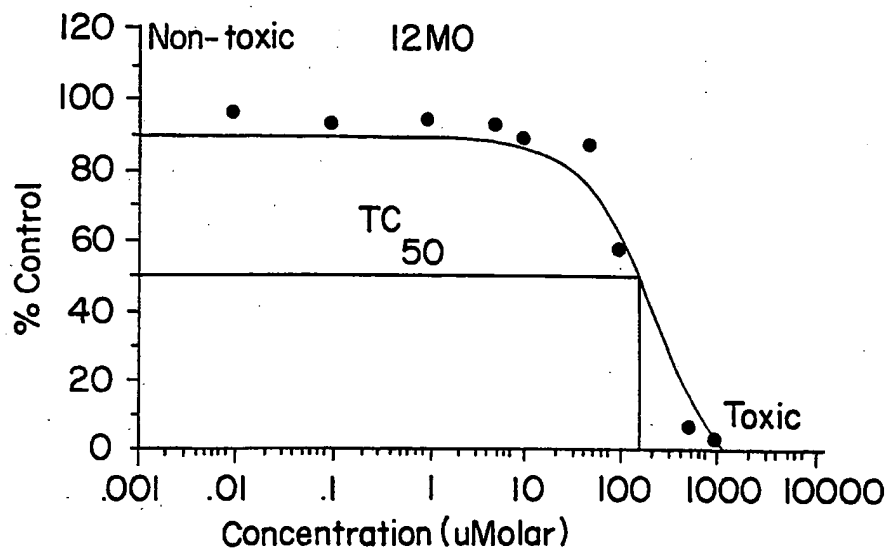


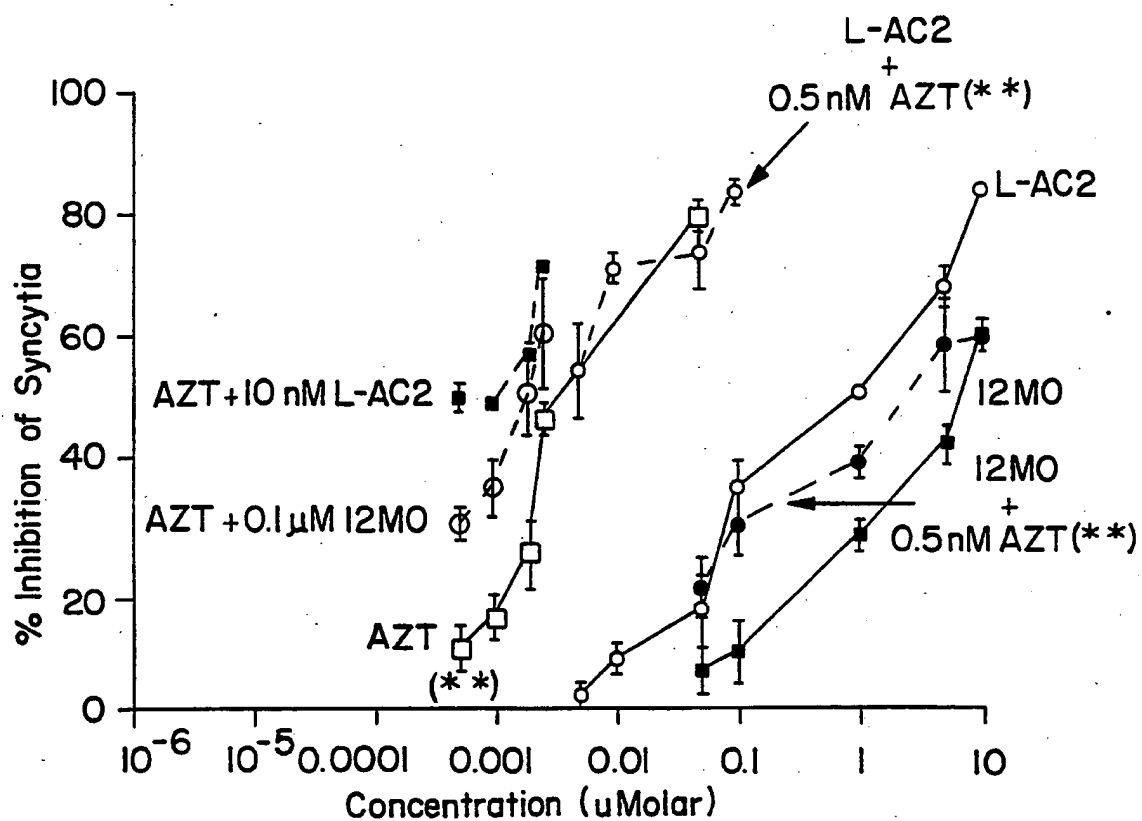
FIG. 5B



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FIG. 6

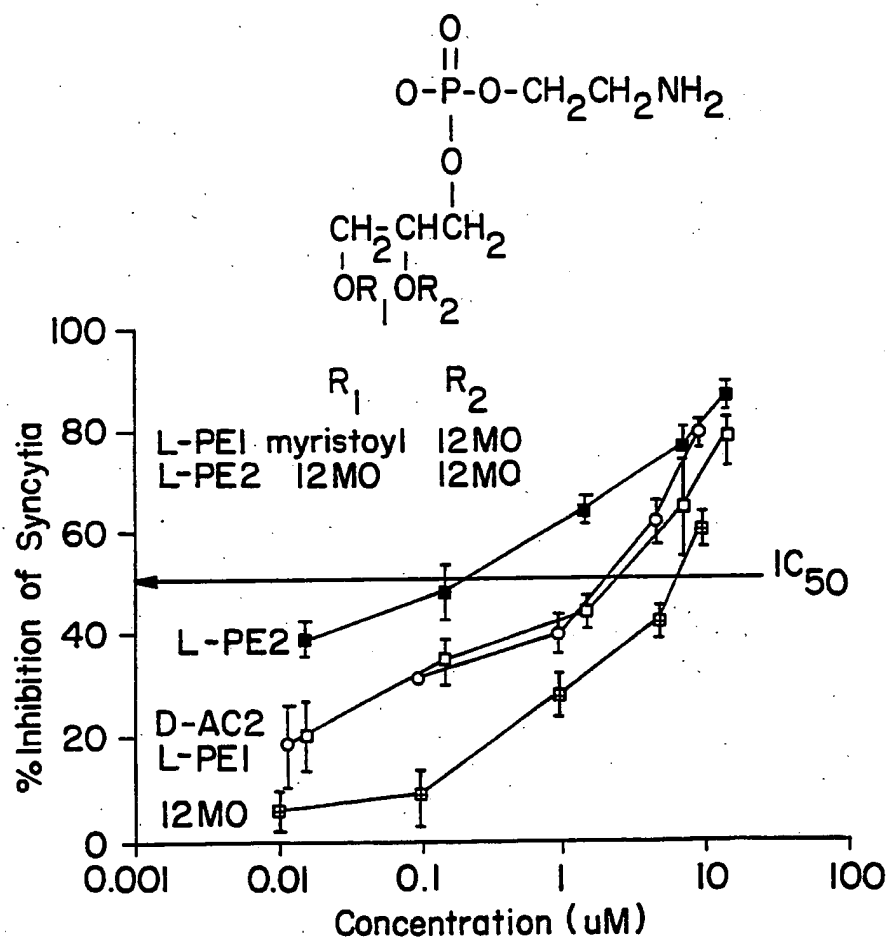


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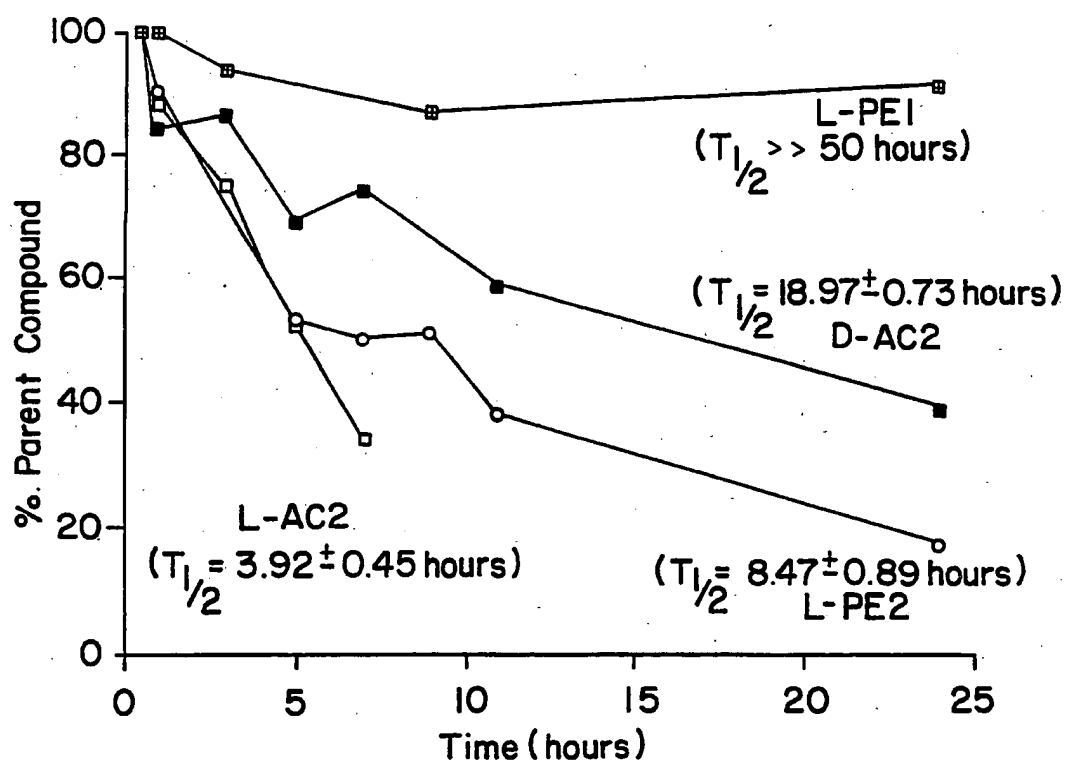
FIG. 7



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FIG. 8



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## INTERNATIONAL SEARCH REPORT

PCT/US 93/03650

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07F9/10; A61K31/66; C07F9/117		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07F ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,0 415 902 (WASHINGTON UNIVERSITY) 6 March 1991 cited in the application see the whole document ---	1,41-44
A	EP,A,0 316 117 (SCIENSCOPE INTERNATIONAL N.V.) 17 May 1989 see the whole document -----	1,41-44
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18 JUNE 1993	- 5. 07. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	BESLIER L.M.	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9303650  
SA 73327

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

18/06/93

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		CA-A- 2024489	02-03-91
		JP-A- 3109322	09-05-91
EP-A-0316117	17-05-89	AU-A- 2619988	01-06-89
		JP-T- 2502096	12-07-90
		WO-A- 8904314	18-05-89